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(54) Title: SUBCELLULAR TARGETING OF THERAPEUTIC PROTEINS

(57) Abstract: Targeted therapeutics that localize to a specific subcellular compartment such as the lysosome are provided. The targeted therapeutics include a therapeutic agent and a targeting moiety that binds a receptor on an exterior surface of the cell, permitting proper subcellular localization of the targeted therapeutic upon internalization of the receptor. Nucleic acids, cells, and methods relating to the practice of the invention are also provided.

SUBCELLULAR TARGETING OF THERAPEUTIC PROTEINS

[0001] This application claims the benefit of U.S. Serial No. 60/287,531, filed April 30, 2001; U.S. Serial No. 60/304,609, filed July 10, 2001; U.S. Serial No. 60/329,461, filed October 15, 2001, and U.S. Serial No. 60/351,276, filed January 23, 2002, the contents of which are incorporated by reference.

[0002] This invention provides a means for specifically delivering proteins to a targeted subcellular compartment of a mammalian cell. The ability to target proteins to a subcellular compartment is of great utility in the treatment of metabolic diseases such as lysosomal storage diseases, a class of over 40 inherited disorders in which particular lysosomal enzymes are absent or deficient.

Background

[0003] Enzyme deficiencies in cellular compartments such as the golgi, the endoplasmic reticulum, and the lysosome cause a wide variety of human diseases. For example, lysyl hydroxylase, an enzyme normally in the lumen of the endoplasmic reticulum, is required for proper processing of collagen; absence of the enzyme causes Ehlers-Danlos syndrome type VI, a serious connective tissue disorder. GnT II, normally found in the golgi, is required for normal glycosylation of proteins; absence of GnT II causes leads to defects in brain development. More than forty lysosomal storage diseases (LSDs) are caused, directly or indirectly, by the absence of one or more proteins in the lysosome.

[0004] Mammalian lysosomal enzymes are synthesized in the cytosol and traverse the ER where they are glycosylated with N-linked, high mannose type carbohydrate. In the golgi, the high mannose carbohydrate is modified on lysosomal proteins by the addition of mannose-6-phosphate (M6P) which targets these proteins to the lysosome. The M6P-

modified proteins are delivered to the lysosome via interaction with either of two M6P receptors. The most favorable form of modification is when two M6Ps are added to a high mannose carbohydrate.

[0005] Enzyme replacement therapy for lysosomal storage diseases (LSDs) is being actively pursued. Therapy, except in Gaucher's disease, generally requires that LSD proteins be taken up and delivered to the lysosomes of a variety of cell types in an M6P-dependent fashion. One possible approach involves purifying an LSD protein and modifying it to incorporate a carbohydrate moiety with M6P. This modified material may be taken up by the cells more efficiently than unmodified LSD proteins due to interaction with M6P receptors on the cell surface. However, because of the time and expense required to prepare, purify and modify proteins for use in subcellular targeting, a need for new, simpler, more efficient, and more cost-effective methods for targeting therapeutic agents to a cellular compartment remains.

Summary of the Invention

[0006] The present invention facilitates the treatment of metabolic diseases by providing targeted protein therapeutics that localize to a subcellular compartment of a cell where the therapeutic is needed. The invention simplifies preparation of targeted protein therapeutics by reducing requirements for posttranslational or postsynthesis processing of the protein. For example, a targeted therapeutic of the present invention can be synthesized as a fusion protein including a therapeutic domain and a domain that targets the fusion protein to a correct subcellular compartment. ("Fusion protein," as used herein, refers to a single polypeptide having at least two domains that are not normally present in the same polypeptide. Thus, naturally occurring proteins are not "fusion proteins" as used herein.) Synthesis as a fusion protein permits targeting of the therapeutic domain to a desired

subcellular compartment without complications associated with chemical crosslinking of separate therapeutic and targeting domains, for example.

[0007] The invention also permits targeting of a therapeutic to a lysosome in an M6P-independent manner. Accordingly, the targeted therapeutic need not be synthesized in a mammalian cell, but can be synthesized chemically or in a bacterium, yeast, protozoan, or other organism regardless of glycosylation pattern, facilitating production of the targeted therapeutic with high yield and comparatively low cost. The targeted therapeutic can be synthesized as a fusion protein, further simplifying production, or can be generated by associating independently-synthesized therapeutic agents and targeting moieties.

[0008] The present invention permits lysosomal targeting of therapeutics without the need for M6P addition to high mannose carbohydrate. It is based in part on the observation that one of the 2 M6P receptors also binds other ligands with high affinity. For example, the cation-independent mannose-6-phosphate receptor is also known as the insulin-like growth factor 2 (IGF-II) receptor because it binds IGF-II with high affinity. This low molecular weight polypeptide interacts with three receptors, the insulin receptor, the IGF-I receptor and the M6P/IGF-II receptor. It is believed to exert its biological effect primarily through interactions with the former two receptors while interaction with the cation-independent M6P receptor is believed to result predominantly in the IGF-II being transported to the lysosome where it is degraded.

[0009] Accordingly, the invention relates in one aspect to a targeted therapeutic including a targeting moiety and a therapeutic agent that is therapeutically active in a mammalian lysosome. "Therapeutically active," as used herein, encompasses at least polypeptides or other molecules that provide an enzymatic activity to a cell or a compartment thereof that is deficient in that activity. "Therapeutically active" also encompasses other polypeptides or other molecules that are intended to ameliorate or to compensate for a

biochemical deficiency in a cell, but does not encompass molecules that are primarily cytotoxic or cytostatic, such as chemotherapeutics.

[0010] In one embodiment, the targeting moiety is a means (*e.g.* a molecule) for binding the extracellular domain of the human cation-independent M6P receptor in an M6P-independent manner when the receptor is present in the plasma membrane of a target cell. In another embodiment, the targeting moiety is an unglycosylated lysosomal targeting domain that binds the extracellular domain of the human cation-independent M6P receptor. In either embodiment, the targeting moiety can include, for example, IGF-II; retinoic acid or a derivative thereof; a protein having an amino acid sequence at least 70% identical to a domain of urokinase-type plasminogen activator receptor; an antibody variable domain that recognizes the receptor; or variants thereof. In some embodiments, the targeting moiety binds to the receptor with a submicromolar dissociation constant (*e.g.* less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, or between 10^{-7} M and 10^{-11} M) at or about pH 7.4 and with an dissociation constant at or about pH 5.5 of at least 10^{-6} M and at least ten times the dissociation constant at or about pH 7.4. In particular embodiments, the means for binding binds to the extracellular domain at least 10-fold less avidly (*i.e.* with at least a ten-fold greater dissociation constant) at or about pH 5.5 than at or about pH 7.4; in one embodiment, the dissociation constant at or about pH 5.5 is at least 10^{-6} M. In a further embodiment, association of the targeted therapeutic with the means for binding is destabilized by a pH change from at or about pH 7.4 to at or about pH 5.5.

[0011] In another embodiment, the targeting moiety is a lysosomal targeting domain that binds the extracellular domain of the human cation-independent M6P receptor but does not bind a mutein of the receptor in which amino acid 1572 is changed from isoleucine to threonine, or binds the mutein with at least ten-fold less affinity (*i.e.* with at least a ten-fold greater dissociation constant). In another embodiment, the targeting moiety is

a lysosomal targeting domain capable of binding a receptor domain consisting essentially of repeats 10-15 of the human cation-independent M6P receptor: the lysosomal targeting domain can bind a protein that includes repeats 10-15 even if the protein includes no other moieties that bind the lysosomal targeting domain. Preferably, the lysosomal targeting domain can bind a receptor domain consisting essentially of repeats 10-13 of the human cation-independent mannose-6-phosphate receptor. More preferably, the lysosomal targeting domain can bind a receptor domain consisting essentially of repeats 11-12, repeat 11, or amino acids 1508-1566 of the human cation-independent M6P receptor. In each of these embodiments, the lysosomal targeting domain preferably binds the receptor or receptor domain with a submicromolar dissociation constant at or about pH 7.4. In one preferred embodiment, the lysosomal targeting domain binds with an dissociation constant of about 10^{-7} M. In another preferred embodiment, the dissociation constant is less than about 10^{-7} M.

[0012] In another embodiment, the targeting moiety is a binding moiety sufficiently duplicative of human IGF-II such that the binding moiety binds the human cation-independent M6P receptor. The binding moiety can be sufficiently duplicative of IGF-II by including an amino acid sequence sufficiently homologous to at least a portion of IGF-II, or by including a molecular structure sufficiently representative of at least a portion of IGF-II, such that the binding moiety binds the cation-independent M6P receptor. The binding moiety can be an organic molecule having a three-dimensional shape representative of at least a portion of IGF-II, such as amino acids 48-55 of human IGF-II, or at least three amino acids selected from the group consisting of amino acids 8, 48, 49, 50, 54, and 55 of human IGF-II. A preferred organic molecule has a hydrophobic moiety at a position representative of amino acid 48 of human IGF-II and a positive charge at or about pH 7.4 at a position representative of amino acid 49 of human IGF-II. In one embodiment, the binding moiety is a polypeptide including a polypeptide having antiparallel alpha-helices separated by

not more than five amino acids. In another embodiment, the binding moiety includes a polypeptide with the amino acid sequence of IGF-I or of a mutein of IGF-I in which amino acids 55-56 are changed and/or amino acids 1-4 are deleted or changed. In a further embodiment, the binding moiety includes a polypeptide with an amino acid sequence at least 60% identical to human IGF-II; amino acids at positions corresponding to positions 54 and 55 of human IGF-II are preferably uncharged or negatively charged at or about pH 7.4.

[0013] In one embodiment, the targeting moiety is a polypeptide comprising the amino acid sequence phenylalanine-arginine-serine. In another embodiment, the targeting moiety is a polypeptide including an amino acid sequence at least 75% homologous to amino acids 48-55 of human IGF-II. In another embodiment, the targeting moiety includes, on a single polypeptide or on separate polypeptides, amino acids 8-28 and 41-61 of human IGF-II. In another embodiment, the targeting moiety includes amino acids 41-61 of human IGF-II and a mutein of amino acids 8-28 of human IGF-II differing from the human sequence at amino acids 9, 19, 26, and/or 27.

[0014] In some embodiments, the association of the therapeutic agent with the targeting moiety is labile at or about pH 5.5. In a preferred embodiment, association of the targeting moiety with the therapeutic agent is mediated by a protein acceptor (such as imidazole or a derivative thereof such as histidine) having a pKa between 5.5 and 7.4. Preferably, one of the therapeutic agent or the targeting moiety is coupled to a metal, and the other is coupled to a pH-dependent metal binding moiety.

[0015] In another aspect, the invention relates to a therapeutic fusion protein including a therapeutic domain and a subcellular targeting domain. The subcellular targeting domain binds to an extracellular domain of a receptor on an exterior surface of a cell. Upon internalization of the receptor, the subcellular targeting domain permits localization of the therapeutic domain to a subcellular compartment such as a lysosome, an endosome, the

endoplasmic reticulum (ER), or the golgi complex, where the therapeutic domain is therapeutically active. In one embodiment, the receptor undergoes constitutive endocytosis. In another embodiment, the therapeutic domain has a therapeutic enzymatic activity. The enzymatic activity is preferably one for which a deficiency (in a cell or in a particular compartment of a cell) is associated with a human disease such as a lysosomal storage disease.

[0016] In further aspects, the invention relates to nucleic acids encoding therapeutic proteins and to cells (*e.g.* mammalian cells, insect cells, yeast cells, protozoans, or bacteria) comprising these nucleic acids. The invention also provides methods of producing the proteins by providing these cells with conditions (*e.g.* in the context of *in vitro* culture or by maintaining the cells in a mammalian body) permitting expression of the proteins. The proteins can be harvested thereafter (*e.g.* if produced *in vitro*) or can be used without an intervening harvesting step (*e.g.* if produced *in vivo* in a patient). Thus, the invention also provides methods of treating a patient by administering a therapeutic protein (*e.g.* by injection, *in situ* synthesis, or otherwise), by administering a nucleic acid encoding the protein (thereby permitting *in vivo* protein synthesis), or by administering a cell comprising a nucleic acid encoding the protein. In one embodiment, the method includes synthesizing a targeted therapeutic including a therapeutic agent that is therapeutically active in a mammalian lysosome and a targeting moiety that binds human cation-independent mannose-6-phosphate receptor in a mannose-6-phosphate-independent manner, and administering the targeted therapeutic to a patient. The method can also include identifying the targeting moiety (*e.g.* by a recombinant display technique such as phage display, bacterial display, or yeast two-hybrid or by screening libraries for requisite binding properties). In another embodiment, the method includes providing (*e.g.* on a computer) a molecular model defining a three-dimensional shape representative of at least a portion of human IGF-II; identifying a

candidate IGF-II analog having a three-dimensional shape representative of at least a portion of IGF-II (*e.g.* amino acids 48-55), and producing a therapeutic agent that is active in a mammalian lysosome and directly or indirectly bound to the candidate IGF-II analog. The method can also include determining whether the candidate IGF-II analog binds to the human cation-independent M6P receptor.

Brief Description of the Drawings

[0017] Figure 1 is a map of the human IGF-II open reading frame. Mature IGF-II lacks the signal peptide and COOH-cleaved regions.

[0018] Figure 2 is a *Leishmania* codon optimized IGF-II depicted in the XbaI site of pIR1-SAT.

[0019] Figure 3 is a depiction of a preferred embodiment of the invention, incorporating a signal peptide sequence, the mature human β-glucuronidase sequence, a bridge of three amino acids, and an IGF-II sequence.

[0020] Figure 4 depicts β-glucuronidase (GUS) activity in human mucopolysaccharidosis VII skin fibroblast GM4668 cells exposed to GUS, a GUS-IGF-II fusion protein (GILT-GUS), GILT-GUS with Δ1-7 and Y27L mutations in the IGF-II portion (GILT^2- GUS), or a negative control (DMEM).

[0021] Figure 5 depicts GUS activity in GM4668 cells exposed to GUS (+β-GUS), GUS-GILT (+GILT), GUS-GILT in the presence of excess IGF-II (+GILT+IGF-II), or a negative control (GM4668).

[0022] Figure 6 is an alignment of human IGF-I and IGF-II, showing the A, B, C, and D domains.

[0023] Figure 7 depicts GUS in GM4668 cells exposed to GUS, GUS-GILT, GUS-GILT, GUS-GILT with a deletion of the seven amino-terminal residues (GUS-GILT

Δ1-7), GUS-GILT in the presence of excess IGF-II, GUS-GILT Δ1-7 in the presence of excess IGF-II; or a negative control (Mock).

Detailed Description of the Invention

[0024] As used herein, "glycosylation independent lysosomal targeting" and "GILT" refer to lysosomal targeting that is mannose-6-phosphate-independent.

[0025] As used herein, "GILT construct" refers to a construct including a mannose-6-phosphate-independent lysosomal targeting portion and a therapeutic portion effective in a mammalian lysosome.

[0026] As used herein, "GUS" refers to ß-glucuronidase, an exemplary therapeutic portion.

[0027] As used herein, "GUS-GILT" refers to a GILT construct with GUS coupled to an IGF-II targeting portion.

[0028] All references to amino acid positions in IGF-II refer to the positions in mature human IGF-II. Thus, for example, positions 1, 2, and 3 are occupied by alanine, tyrosine, and arginine, respectively.

[0029] The present invention facilitates treatment of metabolic diseases by providing targeted therapeutics that, when provided externally to a cell, enter the cell and localize to a subcellular compartment where the targeted therapeutic is active. The targeted therapeutic includes at least a therapeutic agent and a targeting moiety, such as a subcellular targeting domain of a protein, or, for lysosomal targeting, a means (*e.g.* a protein, peptide, peptide analog, or organic chemical) for binding the human cation-independent mannose-6-phosphate receptor.

Association between therapeutic agent and targeting moiety

[0030] The therapeutic agent and the targeting moiety are necessarily associated, directly or indirectly. In one embodiment, the therapeutic agent and the targeting moiety are non-covalently associated. The association is preferably stable at or about pH 7.4. For example, the targeting moiety can be biotinylated and bind avidin associated with the therapeutic agent. Alternatively, the targeting moiety and the therapeutic agent can each be associated (*e.g.* as fusion proteins) with different subunits of a multimeric protein. In another embodiment, the targeting moiety and the therapeutic agent are crosslinked to each other (*e.g.* using a chemical crosslinking agent).

[0031] In a preferred embodiment, the therapeutic agent is fused to the targeting moiety as a fusion protein. The targeting moiety can be at the amino-terminus of the fusion protein, the carboxy-terminus, or can be inserted within the sequence of the therapeutic agent at a position where the presence of the targeting moiety does not unduly interfere with the therapeutic activity of the therapeutic agent.

[0032] Where the therapeutic agent is a heteromeric protein, one or more of the subunits can be associated with a targeting portion. Hexosaminidase A, for example, a lysosomal protein affected in Tay-Sachs disease, includes an alpha subunit and a beta subunit. The alpha subunit, the beta subunit, or both can be associated with a targeting moiety in accordance with the present invention. If, for example, the alpha subunit is associated with a targeting moiety and is coexpressed with the beta subunit, an active complex is formed and targeted appropriately (*e.g.* to the lysosome).

[0033] For targeting a therapeutic to the lysosome, the therapeutic agent can be connected to the targeting moiety through an interaction that is disrupted by decreasing the pH from at or about 7.4 to at or about 5.5. The targeting moiety binds a receptor on the exterior of a cell; the selected receptor is one that undergoes endocytosis and passes through the late endosome, which has a pH of about 5.5. Thus, in the late endosome, the therapeutic

agent dissociates from the targeting moiety and proceeds to the lysosome, where the therapeutic agent acts. For example, a targeting moiety can be chemically modified to incorporate a chelating agent (*e.g.* EDTA, EGTA, or trinitrilotriacetic acid) that tightly binds a metal ion such as nickel. The targeting moiety (*e.g.* GUS) can be expressed as a fusion protein with a six-histidine tag (*e.g.* at the amino-terminus, at the carboxy-terminus, or in a surface-accessible flexible loop). At or about pH 7.4, the six-histidine tag is substantially deprotonated and binds metal ions such as nickel with high affinity. At or about pH 5.5, the six-histidine tag is substantially protonated, leading to release of the nickel and, consequently, release of the therapeutic agent from the targeting moiety.

Therapeutic agent

[0034] While methods and compositions of the invention are useful for producing and delivering any therapeutic agent to a subcellular compartment, the invention is particularly useful for delivering gene products for treating metabolic diseases.

[0035] Preferred LSD genes are shown in Table 1, and preferred genes associated with golgi or ER defects are shown in Table 2. In a preferred embodiment, a wild-type LSD gene product is delivered to a patient suffering from a defect in the same LSD gene. In alternative embodiments, a functional sequence or species variant of the LSD gene is used. In further embodiments, a gene coding for a different enzyme that can rescue an LSD gene defect is used according to methods of the invention.

Table 1. Lysosomal Storage Diseases and associated enzyme defects

A. Glycogenosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
Pompe Disease	Acid-a1, 4-Glucosidase	Glycogen α 1-4 linked Oligosaccharides
B. Glycolipidosis Disorders		
Disease Name	Enzyme Defect	Substance Stored
GM1 Gangliodsidosis	β-Galactosidase	GM_1 Ganliosides
Tay-Sachs Disease	β-Hexosaminidase A	GM_2 Ganglioside
GM2 Gangliosidosis: AB Variant	GM_2 Activator Protein	GM_2 Ganglioside
Sandhoff Disease	β-Hexosaminidase A&B	GM_2 Ganglioside
Fabry Disease	α-Galactosidase A	Globosides
Gaucher Disease	Glucocerebrosidase	Glucosylceramide
Metachromatic Leukodystrophy	Arylsulfatase A	Sulphatides
Krabbe Disease	Galactosylceramidase	Galactocerebroside
Niemann-Pick, Types A and B	Acid Sphingomyelinase	Sphingomyelin
Niemann-Pick, Type C	Cholesterol Esterification Defect	Sphingomyelin
Nieman-Pick, Type D	Unknown	Sphingomyelin
Farber Disease	Acid Ceramidase	Ceramide
Wolman Disease	Acid Lipase	Cholesteryl Esters
C. Mucopolysaccharide Disorders		
Disease Name	Enzyme Defect	Substance Stored
Hurler Syndrome (MPS IH)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Scheie Syndrome (MPS IS)	α-L-Iduronidase	Heparan & Dermatan, Sulfates
Hurler-Scheie (MPS IH/S)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Hunter Syndrome (MPS II)	Iduronate Sulfatase	Heparan & Dermatan Sulfates
Sanfilippo A (MPS IIIA)	Heparan N-Sulfatase	Heparan Sulfate
Sanfilippo B	α-N-	Heparan

(MPS IIIB)	Acetylglucosaminidase	Sulfate
Sanfilippo C (MPS IIIC)	Acetyl-CoA-Glucosaminide Acetyltransferase	Heparan Sulfate
Sanfilippo D (MPS IIID)	N-Acetylglucosamine -6-Sulfatase	Heparan Sulfate
Morquio A (MPS IVA)	Galactosamine-6-Sulfatase	Keratan Sulfate
Morquio B (MPS IVB)	β-Galactosidase	Keratan Sulfate
Maroteaux-Lamy (MPS VI)	Arylsulfatase B	Dermatan Sulfate
Sly Syndrome (MPS VII)	β-Glucuronidase	
D. Oligosaccharide/Glycoprotein Disorders		
Disease Name	Enzyme Defect	Substance Stored
α–Mannosidosis	α–Mannosidase	Mannose/Oligosaccharides
β-Mannosidosis	β-Mannosidase	Mannose/Oligosaccharides
Fucosidosis	α-L-Fucosidase	Fucosyl Oligosaccharides
Asparylglucosaminuria	N-Aspartyl- β-Glucosaminidase	Asparylglucosamine Asparagines
Sialidosis (Mucolipidosis I)	α-Neuraminidase	Sialyloligosaccharides
Galactosialidosis (Goldberg Syndrome)	Lysosomal Protective Protein Deficiency	Sialyloligosaccharides
Schindler Disease	α-N-Acetyl-Galactosaminidase	
E. Lysosomal Enzyme Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Mucolipidosis II (I-Cell Disease)	N-Acetylglucosamine-1- Phosphotransferase	Heparan Sulfate
Mucolipidosis III (Pseudo-Hurler Polydystrophy)	Same as ML II	

F. Lysosomal Membrane Transport Disorders		
Disease Name	Enzyme Defect	Substance Stored
Cystinosis	Cystine Transport	Free Cystine

	Protein	
Salla Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
Infantile Sialic Acid Storage Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
G. Other		
Disease Name	Enzyme Defect	Substance Stored
Batten Disease (Juvenile Neuronal Ceroid Lipofuscinosis)	Unknown	Lipofuscins
Infantile Neuronal Ceroid Lipofuscinosis	Palmitoyl-Protein Thioesterase	Lipofuscins
Mucolipidosis IV	Unknown	Gangliosides & Hyaluronic Acid
Prosaposin	Saposins A, B, C or D	

Table 2. Diseases of the golgi and ER

Disease Name	Gene and Enzyme Defect	Features
Ehlers-Danlos Syndrome Type VI	PLOD1 lysyl hydroxylase	Defect in lysyl hydroxylation of Collagen; located in ER lumen
Type Ia glycoge storage disease	glucose6 phosphatase	Causes excessive accumulation of Glycogen in the liver, kidney, and Intestinal mucosa; enzyme is transmembrane but active site is ER lumen
Congenital Disorders of Glycosylation		
CDG Ic	ALG6 α1,3 glucosyltransferase	Defects in N-glycosylation ER lumen
CDG Id	ALG3 α1,3 mannosyltransferase	Defects in N-glycosylation ER transmembrane protein
CDG IIa	MGAT2 N-acetylglucosaminyl-transferase II	Defects in N-glycosylation golgi transmembrane protein
CDG IIb	GCS1 α1,2-Glucosidase I	Defect in N glycosylation ER membrane bound with lumenal catalytic domain releasable by proteolysis

[0036] One particularly preferred therapeutic agent is glucocerebrosidase, currently manufactured by Genzyme as an effective enzyme replacement therapy for Gaucher's Disease. Currently, the enzyme is prepared with exposed mannose residues, which targets the protein specifically to cells of the macrophage lineage. Although the primary pathology in type 1 Gaucher patients are due to macrophage accumulating glucocerebroside, there can be therapeutic advantage to delivering glucocerebrosidase to other cell types. Targeting glucocerebrosidase to lysosomes using the present invention would target the agent to multiple cell types and can have a therapeutic advantage compared to other preparations.

Subcellular targeting domains

[0037] The present invention permits targeting of a therapeutic agent to a lysosome using a protein, or an analog of a protein, that specifically binds a cellular receptor for that protein. The exterior of the cell surface is topologically equivalent to endosomal, lysosomal, golgi, and endoplasmic reticulum compartments. Thus, endocytosis of a molecule through interaction with an appropriate receptor(s) permits transport of the molecule to any of these compartments without crossing a membrane. Should a genetic deficiency result in a deficit of a particular enzyme activity in any of these compartments, delivery of a therapeutic protein can be achieved by tagging it with a ligand for the appropriate receptor(s).

[0038] Multiple pathways directing receptor-bound proteins from the plasma membrane to the golgi and/or endoplasmic reticulum have been characterized. Thus, by using a targeting portion from, for example, SV40, cholera toxin, or the plant toxin ricin, each of which coopt one or more of these subcellular trafficking pathways, a therapeutic can be targeted to the desired location within the cell. In each case, uptake is initiated by binding of the material to the exterior of the cell. For example, SV40 binds to MHC class I receptors, cholera toxin binds to GM1 ganglioside molecules and ricin binds to glycolipids and glycoproteins with terminal galactose on the surface of cells. Following this initial step the molecules reach the ER by a variety of pathways. For example, SV40 undergoes caveolar endocytosis and reaches the ER in a two step process that bypasses the golgi whereas cholera toxin undergoes caveolar endocytosis but traverses the golgi before reaching the ER.

[0039] If a targeting moiety related to cholera toxin or ricin is used, it is important that the toxicity of cholera toxin or ricin be avoided. Both cholera toxin and ricin are heteromeric proteins, and the cell surface binding domain and the catalytic activities responsible for toxicity reside on separate polypeptides. Thus, a targeting moiety can be constructed that includes the receptor-binding polypeptide, but not the polypeptide responsible for toxicity. For example, in the case of ricin, the B subunit possesses the

galactose binding activity responsible for internalization of the protein, and can be fused to a therapeutic protein. If the further presence of the A subunit improves subcellular localization, a mutant version (mutein) of the A chain that is properly folded but catalytically inert can be provided with the B subunit-therapeutic agent fusion protein.

[0040] Proteins delivered to the golgi can be transported to the endoplasmic reticulum (ER) via the KDEL receptor, which retrieves ER-targeted proteins that have escaped to the golgi. Thus, inclusion of a KDEL motif at the terminus of a targeting domain that directs a therapeutic protein to the golgi permits subsequent localization to the ER. For example, a targeting moiety (*e.g.* an antibody, or a peptide identified by high-throughput screening such as phage display, yeast two hybrid, chip-based assays, and solution-based assays) that binds the cation-independent M6P receptor both at or about pH 7.4 and at or about pH 5.5 permits targeting of a therapeutic agent to the golgi; further addition of a KDEL motif permits targeting to the ER.

Lysosomal targeting moieties

[0041] The invention permits targeting of a therapeutic agent to a lysosome. Targeting may occur, for example, through binding of a plasma membrane receptor that later passes through a lysosome. Alternatively, targeting may occur through binding of a plasma receptor that later passes through a late endosome; the therapeutic agent can then travel from the late endosome to a lysosome. A preferred lysosomal targeting mechanism involves binding to the cation-independent M6P receptor.

Cation-independent M6P receptor

[0042] The cation-independent M6P receptor is a 275 kDa single chain transmembrane glycoprotein expressed ubiquitously in mammalian tissues. It is one of two mammalian receptors that bind M6P: the second is referred to as the cation-dependent M6P receptor. The cation-dependent M6P receptor requires divalent cations for M6P binding; the

cation-independent M6P receptor does not. These receptors play an important role in the trafficking of lysosomal enzymes through recognition of the M6P moiety on high mannose carbohydrate on lysosomal enzymes. The extracellular domain of the cation-independent M6P receptor contains 15 homologous domains ("repeats") that bind a diverse group of ligands at discrete locations on the receptor.

[0043] The cation-independent M6P receptor contains two binding sites for M6P: one located in repeats 1-3 and the other located in repeats 7-9. The receptor binds monovalent M6P ligands with a dissociation constant in the μM range while binding divalent M6P ligands with a dissociation constant in the nM range, probably due to receptor oligomerization. Uptake of IGF-II by the receptor is enhanced by concomitant binding of multivalent M6P ligands such as lysosomal enzymes to the receptor.

[0044] The cation-independent M6P receptor also contains binding sites for at least three distinct ligands that can be used as targeting moieties. The cation-independent M6P receptor binds IGF-II with a dissociation constant of about 14 nM at or about pH 7.4, primarily through interactions with repeat 11. Consistent with its function in targeting IGF-II to the lysosome, the dissociation constant is increased approximately 100-fold at or about pH 5.5 promoting dissociation of IGF-II in acidic late endosomes. The receptor is capable of binding high molecular weight O-glycosylated IGF-II forms.

[0045] An additional useful ligand for the cation-independent M6P receptor is retinoic acid. Retinoic acid binds to the receptor with a dissociation constant of 2.5 nM. Affinity photolabeling of the cation-independent M6P receptor with retinoic acid does not interfere with IGF-II or M6P binding to the receptor, indicating that retinoic acid binds to a distinct site on the receptor. Binding of retinoic acid to the receptor alters the intracellular distribution of the receptor with a greater accumulation of the receptor in cytoplasmic vesicles and also enhances uptake of M6P modified ß-glucuronidase. Retinoic acid has a

photoactivatable moiety that can be used to link it to a therapeutic agent without interfering with its ability to bind to the cation-independent M6P receptor.

[0046] The cation-independent M6P receptor also binds the urokinase-type plasminogen receptor (uPAR) with a dissociation constant of 9 μM. uPAR is a GPI-anchored receptor on the surface of most cell types where it functions as an adhesion molecule and in the proteolytic activation of plasminogen and TGF-ß. Binding of uPAR to the CI-M6P receptor targets it to the lysosome, thereby modulating its activity. Thus, fusing the extracellular domain of uPAR, or a portion thereof competent to bind the cation-independent M6P receptor, to a therapeutic agent permits targeting of the agent to a lysosome.

IGF-II

[0047] In a preferred embodiment, the lysosomal targeting portion is a protein, peptide, or other moiety that binds the cation independent M6P/IGF-II receptor in a mannose-6-phosphate-independent manner. Advantageously, this embodiment mimics the normal biological mechanism for uptake of LSD proteins, yet does so in a manner independent of mannose-6-phosphate.

[0048] For example, by fusing DNA encoding the mature IGF-II polypeptide to the 3' end of LSD gene cassettes, fusion proteins are created that can be taken up by a variety of cell types and transported to the lysosome. This method has numerous advantages over methods involving glycosylation including simplicity and cost effectiveness, because once the protein is isolated, no further modifications need be made.

[0049] IGF-II is preferably targeted specifically to the M6P receptor. Particularly useful are mutations in the IGF-II polypeptide that result in a protein that binds the M6P receptor with high affinity while no longer binding the other two receptors with appreciable affinity. IGF-II can also be modified to minimize binding to serum IGF-binding proteins

(Baxter (2000) Am. J. Physiol Endocrinol Metab. 278(6):967-76) to avoid sequestration of IGF-II/GILT constructs. A number of studies have localized residues in IGF-1 and IGF-II necessary for binding to IGF-binding proteins. Constructs with mutations at these residues can be screened for retention of high affinity binding to the M6P/IGF-II receptor and for reduced affinity for IGF-binding proteins. For example, replacing PHE 26 of IGF-II with SER is reported to reduce affinity of IGF-II for IGFBP-1 and -6 with no effect on binding to the M6P/IGF-II receptor (Bach *et al.* (1993) J. Biol. Chem. 268(13):9246-54). Other substitutions, such as SER for PHE 19 and LYS for GLU 9, can also be advantageous. The analogous mutations, separately or in combination, in a region of IGF-I that is highly conserved with IGF-II result in large decreases in IGF-BP binding (Magee *et al.* (1999) Biochemistry 38(48):15863-70).

[0050] An alternate approach is to identify minimal regions of IGF-II that can bind with high affinity to the M6P/IGF-II receptor. The residues that have been implicated in IGF-II binding to the M6P/IGF-II receptor mostly cluster on one face of IGF-II (Terasawa *et al.* (1994) EMBO J. 13(23):5590-7). Although IGF-II tertiary structure is normally maintained by three intramolecular disulfide bonds, a peptide incorporating the amino acid sequence on the M6P/IGF-II receptor binding surface of IGF-II can be designed to fold properly and have binding activity. Such a minimal binding peptide is a highly preferred targeting portion. Designed peptides based on the region around amino acids 48-55 can be tested for binding to the M6P/IGF-II receptor. Alternatively, a random library of peptides can be screened for the ability to bind the M6P/IGF-II receptor either via a yeast two hybrid assay, or via a phage display type assay.

Blood brain barrier

[0051] One challenge in therapy for lysosomal storage diseases is that many of these diseases have significant neurological involvement. Therapeutic enzymes administered

into the blood stream generally do not cross the blood brain barrier and therefore cannot relieve neurological symptoms associated with the diseases. IGF-II, however, has been reported to promote transport across the blood brain barrier via transcytosis (Bickel *et al.* (2001) Adv. Drug Deliv. Rev. 46(1-3):247-79). Thus, appropriately designed GILT constructs should be capable of crossing the blood brain barrier, affording for the first time a means of treating neurological symptoms associated with lysosomal storage diseases. The constructs can be tested using GUS minus mice as described in Example 7, *infra*. Further details regarding design, construction and testing of targeted therapeutics that can reach neuronal tissue from blood are disclosed in U.S. Serial No. 60/329,650, filed October 16, 2001, and in U.S. Serial No. 10/___,___ (Attorney Docket No. SYM-008), filed April 30, 2002.

Structure of IGF-II

[0052] NMR structures of IGF-II have been solved by two groups (Terasawa *et al.* (1994) EMBO J. 13(23):5590-7; Torres *et al.* (1995) J. Mol. Biol. 248(2):385-401) (see, *e.g.*, Protein Data Bank record 1IGL). The general features of the IGF-II structure are similar to IGF-I and insulin. The A and B domains of IGF-II correspond to the A and B chains of insulin. Secondary structural features include an alpha helix from residues 11-21 of the B region connected by a reverse turn in residues 22-25 to a short beta strand in residues 26-28. Residues 25-27 appear to form a small antiparallel beta sheet; residues 59-61 and residues 26-28 may also participate in intermolecular beta-sheet formation. In the A domain of IGF-II, alpha helices spanning residues 42-49 and 53-59 are arranged in an antiparallel configuration perpendicular to the B-domain helix. Hydrophobic clusters formed by two of the three disulfide bridges and conserved hydrophobic residues stabilize these secondary structure features. The N and C termini remain poorly defined as is the region between residues 31-40.

[0053] IGF-II binds to the IGF-II/M6P and IGF-I receptors with relatively high affinity and binds with lower affinity to the insulin receptor. IGF-II also interacts with a number if serum IGFBPs.

Binding to the IGF-II/M6P receptor

[0054] Substitution of IGF-II residues 48-50 (Phe Arg Ser) with the corresponding residues from insulin, (Thr Ser Ile), or substitution of residues 54-55 (Ala Leu) with the corresponding residues from IGF-I (Arg Arg) result in diminished binding to the IGF-II/ M6P receptor but retention of binding to the IGF-I and insulin receptors (Sakano *et al.* (1991) J. Biol. Chem. 266(31):20626-35).

[0055] IGF-I and IGF-II share identical sequences and structures in the region of residues 48-50 yet have a 1000-fold difference in affinity for the IGF-II receptor. The NMR structure reveals a structural difference between IGF-I and IGF-II in the region of IGF-II residues 53-58 (IGF-I residues 54-59): the alpha-helix is better defined in IGF-II than in IGF-I and, unlike IGF-I, there is no bend in the backbone around residues 53 and 54 (Torres *et al.* (1995) J. Mol. Biol. 248(2):385-401). This structural difference correlates with the substitution of Ala 54 and Leu 55 in IGF-II with Arg 55 and Arg 56 in IGF-I. It is possible either that binding to the IGF-II receptor is disrupted directly by the presence of charged residues in this region or that changes in the structure engendered by the charged residues yield the changes in binding for the IGF-II receptor. In any case, substitution of uncharged residues for the two Arg residues in IGF-I resulted in higher affinities for the IGF-II receptor (Cacciari *et al.* (1987) Pediatrician 14(3):146-53). Thus the presence of positively charged residues in these positions correlates with loss of binding to the IGF-II receptor.

[0056] IGF-II binds to repeat 11 of the cation-independent M6P receptor. Indeed, a minireceptor in which only repeat 11 is fused to the transmembrane and cytoplasmic domains of the cation-independent M6P receptor is capable of binding IGF-II (with an

affinity approximately one tenth the affinity of the full length receptor) and mediating internalization of IGF-II and its delivery to lysosomes (Grimme *et al.* (2000) J. Biol. Chem. 275(43):33697-33703). The structure of domain 11 of the M6P receptor is known (Protein Data Base entries 1GP0 and 1GP3; Brown *et al.* (2002) EMBO J. 21(5):1054-1062). The putative IGF-II binding site is a hydrophobic pocket believed to interact with hydrophobic amino acids of IGF-II; candidate amino acids of IGF-II include leucine 8, phenylalanine 48, alanine 54, and leucine 55. Although repeat 11 is sufficient for IGF-II binding, constructs including larger portions of the cation-independent M6P receptor (*e.g.* repeats 10-13, or 1-15) generally bind IGF-II with greater affinity and with increased pH dependence (see, for example, Linnell *et al.* (2001) J. Biol. Chem. 276(26):23986-23991).

Binding to the IGF-I receptor

[0057] Substitution of IGF-II residues Tyr 27 with Leu, Leu 43 with Val or Ser 26 with Phe diminishes the affinity of IGF-II for the IGF-I receptor by 94-, 56-, and 4-fold respectively (Torres *et al.* (1995) J. Mol. Biol. 248(2):385-401). Deletion of residues 1-7 of human IGF-II resulted in a 30-fold decrease in affinity for the human IGF-I receptor and a concomitant 12 fold increase in affinity for the rat IGF-II receptor (Hashimoto *et al.* (1995) J. Biol. Chem. 270(30):18013-8). The NMR structure of IGF-II shows that Thr 7 is located near residues 48 Phe and 50 Ser as well as near the 9 Cys-47 Cys disulfide bridge. It is thought that interaction of Thr 7 with these residues can stabilize the flexible N-terminal hexapeptide required for IGF-I receptor binding (Terasawa *et al.* (1994) EMBO J. 13(23)5590-7). At the same time this interaction can modulate binding to the IGF-II receptor. Truncation of the C-terminus of IGF-II (residues 62-67) also appear to lower the affinity of IGF-II for the IGF-I receptor by 5 fold (Roth *et al.* (1991) Biochem. Biophys. Res. Commun. 181(2):907-14).

Deletion mutants of IGF-II

[0058] The binding surfaces for the IGF-I and cation-independent M6P receptors are on separate faces of IGF-II. Based on structural and mutational data, functional cation-independent M6P binding domains can be constructed that are substantially smaller than human IGF-II. For example, the amino terminal amino acids 1-7 and/or the carboxy terminal residues 62-67 can be deleted or replaced. Additionally, amino acids 29-40 can likely be eliminated or replaced without altering the folding of the remainder of the polypeptide or binding to the cation-independent M6P receptor. Thus, a targeting moiety including amino acids 8-28 and 41-61 can be constructed. These stretches of amino acids could perhaps be joined directly or separated by a linker. Alternatively, amino acids 8-28 and 41-61 can be provided on separate polypeptide chains. Comparable domains of insulin, which is homologous to IGF-II and has a tertiary structure closely related to the structure of IGF-II, have sufficient structural information to permit proper refolding into the appropriate tertiary structure, even when present in separate polypeptide chains (Wang *et al.* (1991) Trends Biochem. Sci. 279-281). Thus, for example, amino acids 8-28, or a conservative substitution variant thereof, could be fused to a therapeutic agent; the resulting fusion protein could be admixed with amino acids 41-61, or a conservative substitution variant thereof, and administered to a patient.

Binding to IGF Binding proteins

[0059] IGF-II and related constructs can be modified to diminish their affinity for IGFBPs, thereby increasing the bioavailability of the tagged proteins.

[0060] Substitution of IGF-II residue phenylalanine 26 with serine reduces binding to IGFBPs 1-5 by 5-75 fold (Bach *et al.* (1993) J. Biol. Chem. 268(13):9246-54). Replacement of IGF-II residues 48-50 with threonine-serine-isoleucine reduces binding by more than 100 fold to most of the IGFBPs (Bach *et al.* (1993) J. Biol. Chem. 268(13):9246-

54); these residues are, however, also important for binding to the cation-independent mannose-6-phosphate receptor. The Y27L substitution that disrupts binding to the IGF-I receptor interferes with formation of the ternary complex with IGFBP3 and acid labile subunit (Hashimoto *et al.* (1997) J. Biol. Chem. 272(44):27936-42); this ternary complex accounts for most of the IGF-II in the circulation (Yu *et al.* (1999) J. Clin. Lab Anal. 13(4):166-72). Deletion of the first six residues of IGF-II also interferes with IGFBP binding (Luthi *et al.* (1992) Eur. J. Biochem. 205(2):483-90).

[0061] Studies on IGF-I interaction with IGFBPs revealed additionally that substitution of serine for phenylalanine 16 did not effect secondary structure but decreased IGFBP binding by between 40 and 300 fold (Magee *et al.* (1999) Biochemistry 38(48):15863-70). Changing glutamate 9 to lysine also resulted in a significant decrease in IGFBP binding. Furthermore, the double mutant lysine 9/ serine 16 exhibited the lowest affinity for IGFBPs. Although these mutations have not previously been tested in IGF-II, the conservation of sequence between this region of IGF-I and IGF-II suggests that a similar effect will be observed when the analogous mutations are made in IGF-II (glutamate 12 lysine/ phenylalanine 19 serine).

IGF-II homologs

[0062] The amino acid sequence of human IGF-II, or a portion thereof affecting binding to the cation-independent M6P receptor, may be used as a reference sequence to determine whether a candidate sequence possesses sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. Preferably, variant sequences are at least 70% similar or 60% identical, more preferably at least 75% similar or 65% identical, and most preferably 80% similar or 70% identical to human IGF-II.

[0063] To determine whether a candidate peptide region has the requisite percentage similarity or identity to human IGF-II, the candidate amino acid sequence and

human IGF-II are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981) J. Mol. Biol. 147:195-197, in combination with the BLOSUM62 substitution matrix described in Figure 2 of Henikoff and Henikoff (1992) PNAS 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is -12, and an appropriate value for the gap extension penalty is -4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

[0064] Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pairwise similarity score is zero; otherwise the pairwise similarity score is 1.0. The raw similarity score is the sum of the pairwise similarity scores of the aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence are again compared sequentially. If the amino acids are non-identical, the pairwise identity score is zero; otherwise the pairwise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used in the initial alignment.

IGF-II structural analogs

[0065] The known structures of human IGF-II and the cation-independent M6P receptors permit the design of IGF-II analogs and other cation-independent M6P receptor binding proteins using computer-assisted design principles such as those discussed in U.S. Patent Nos. 6,226,603 and 6,273,598. For example, the known atomic coordinates of IGF-II can be provided to a computer equipped with a conventional computer modeling program, such as INSIGHTII, DISCOVER, or DELPHI, commercially available from Biosym, Technologies Inc., or QUANTA, or CHARMM, commercially available from Molecular Simulations, Inc. These and other software programs allow analysis of molecular structures and simulations that predict the effect of molecular changes on structure and on intermolecular interactions. For example, the software can be used to identify modified analogs with the ability to form additional intermolecular hydrogen or ionic bonds, improving the affinity of the analog for the target receptor.

[0066] The software also permits the design of peptides and organic molecules with structural and chemical features that mimic the same features displayed on at least part of the surface of the cation-independent M6P receptor binding face of IGF-II. Because a major contribution to the receptor binding surface is the spatial arrangement of chemically interactive moieties present within the sidechains of amino acids which together define the receptor binding surface, a preferred embodiment of the present invention relates to designing and producing a synthetic organic molecule having a framework that carries chemically interactive moieties in a spatial relationship that mimics the spatial relationship of the chemical moieties disposed on the amino acid sidechains which constitute the cation-independent M6P receptor binding face of IGF-II. Preferred chemical moieties, include but are not limited to, the chemical moieties defined by the amino acid side chains of amino acids constituting the cation-independent M6P receptor binding face of IGF-II. It is understood,

therefore, that the receptor binding surface of the IGF-II analog need not comprise amino acid residues but the chemical moieties disposed thereon.

[0067] For example, upon identification of relevant chemical groups, the skilled artisan using a conventional computer program can design a small molecule having the receptor interactive chemical moieties disposed upon a suitable carrier framework. Useful computer programs are described in, for example, Dixon (1992) Tibtech 10: 357-363; Tschinke et al. (1993) J. Med. Chem 36: 3863-3870; and Eisen el al. (1994) Proteins: Structure, Function, and Genetics 19: 199-221, the disclosures of which are incorporated herein by reference.

[0068] One particular computer program entitled "CAVEAT" searches a database, for example, the Cambridge Structural Database, for structures which have desired spatial orientations of chemical moieties (Bartlett et al. (1989) in "Molecular Recognition: Chemical and Biological Problems" (Roberts, S. M., ed) pp 182-196). The CAVEAT program has been used to design analogs of tendamistat, a 74 residue inhibitor of α-amylase, based on the orientation of selected amino acid side chains in the three-dimensional structure of tendamistat (Bartlett et al. (1989) supra).

[0069] Alternatively, upon identification of a series of analogs which mimic the cation-independent M6P receptor binding activity of IGF-II, the skilled artisan may use a variety of computer programs which assist the skilled artisan to develop quantitative structure activity relationships (QSAR) and further to assist in the de novo design of additional morphogen analogs. Other useful computer programs are described in, for example, Connolly-Martin (1991) Methods in Enzymology 203:587-613; Dixon (1992) supra; and Waszkowycz et al. (1994) J. Med. Chenm. 37: 3994-4002.

Targeting moiety affinities

[0070] Preferred targeting moieties bind to their target receptors with a submicromolar dissociation constant. Generally speaking, lower dissociation constants (*e.g.* less than 10^{-7} M, less than 10^{-8} M, or less than 10^{-9} M) are increasingly preferred. Determination of dissociation constants is preferably determined by surface plasmon resonance as described in Linnell *et al.* (2001) J. Biol. Chem. 276(26):23986-23991. A soluble form of the extracellular domain of the target receptor (*e.g.* repeats 1-15 of the cation-independent M6P receptor) is generated and immobilized to a chip through an avidin-biotin interaction. The targeting moiety is passed over the chip, and kinetic and equilibrium constants are detected and calculated by measuring changes in mass associated with the chip surface.

Nucleic acids and expression systems

[0071] Chimeric fusion proteins can be expressed in a variety of expression systems, including *in vitro* translation systems and intact cells. Since M6P modification is not a prerequisite for targeting, a variety of expression systems including yeast, baculovirus and even prokaryotic systems such as E. coli that do not glycosylate proteins are suitable for expression of targeted therapeutic proteins. In fact, an unglycosylated protein generally has improved bioavailability, since glycosylated proteins are rapidly cleared from the circulation through binding to the mannose receptor in hepatic sinusoidal endothelium.

[0072] Alternatively, production of chimeric targeted lysosomal enzymes in mammalian cell expression system produces proteins with multiple binding determinants for the cation-independent M6P receptor. Synergies between two or more cation-independent M6P receptor ligands (*e.g.* M6P and IGF-II, or M6P and retinoic acid) can be exploited:

multivalent ligands have been demonstrated to enhance binding to the receptor by receptor crosslinking.

[0073] In general, gene cassettes encoding the chimeric therapeutic protein can be tailored for the particular expression system to incorporate necessary sequences for optimal expression including promoters, ribosomal binding sites, introns, or alterations in coding sequence to optimize codon usage. Because the protein is preferably secreted from the producing cell, a DNA encoding a signal peptide compatible with the expression system can be substituted for the endogenous signal peptide. For example, for expression of ß-glucuronidase and α-galactosidase A tagged with IGF-II in Leishmania, DNA cassettes encoding Leishmania signal peptides (GP63 or SAP) are inserted in place of the DNA encoding the endogenous signal peptide to achieve optimal expression. In mammalian expression systems the endogenous signal peptide may be employed but if the IGF-II tag is fused at the 5' end of the coding sequence, it could be desirable to use the IGF-II signal peptide.

[0074] CHO cells are a preferred mammalian host for the production of therapeutic proteins. The classic method for achieving high yield expression from CHO cells is to use a CHO cell line deficient in dihydrofolate reductase (DHFR), for example CHO line DUKX (O'Dell *et al.* (1998) Int. J. Biochem. Cell Biol. 30(7):767-71). This strain of CHO cells requires hypoxanthine and thymidine for growth. Co-transfection of the gene to be overexpressed with a DHFR gene cassette, on separate plasmids or on a single plasmid, permits selection for the DHFR gene and generally allows isolation of clones that also express the recombinant protein of choice. For example, plasmid pcDNA3 uses the cytomegalovirus (CMV) early region regulatory region promoter to drive expression of a gene of interest and pSV2DHFR to promote DHFR expression. Subsequent exposure of cells harboring the recombinant gene cassettes to incrementally increasing concentrations of the

folate analog methotrexate leads to amplification of both the gene copy number of the DHFR gene and of the co-transfected gene.

[0075] A preferred plasmid for eukaryotic expression in this system contains the gene of interest placed downstream of a strong promoter such as CMV. An intron can be placed in the 3' flank of the gene cassette. A DHFR cassette can be driven by a second promoter from the same plasmid or from a separate plasmid. Additionally, it can be useful to incorporate into the plasmid an additional selectable marker such as neomycin phosphotransferase, which confers resistance to G418.

[0076] Alternatively, recombinant protein can be produced in the human HEK 293 cell line using expression systems based on the Epstein-Barr Virus (EBV) replication system. This consists of the EBV replication origin oriP and the EBV ori binding protein, EBNA-1. Binding of EBNA-1 to oriP initiates replication and subsequent amplification of the extrachromosomal plasmid. This amplification in turn results in high levels of expression of gene cassettes housed within the plasmid. Plasmids containing *oriP* can be transfected into EBNA-1 transformed HEK 293 cells (commercially available from Invitrogen) or, alternatively, a plasmid such as pCEP4 (commercially available from Invitrogen) which drives expression of EBNA-1 and contains the EBV *oriP* can be employed.

[0077] In *E. coli*, the therapeutic proteins are preferably secreted into the periplasmic space. This can be achieved by substituting for the DNA encoding the endogenous signal peptide of the LSD protein a nucleic acid cassette encoding a bacterial signal peptide such as the ompA signal sequence. Expression can be driven by any of a number of strong inducible promoters such as the *lac*, *trp*, or *tac* promoters. One suitable vector is pBAD/gIII (commercially available from Invitrogen) which uses the Gene III signal peptide and the araBAD promoter.

In vitro refolding

[0078] One useful IGF-II targeting portion has three intramolecular disulfide bonds. GILT fusion proteins (for example GUS-GILT) in E. coli can be constructed that direct the protein to the periplasmic space. IGF-II, when fused to the C-terminus of another protein, can be secreted in an active form in the periplasm of E. coli (Wadensten *et al.* (1991) Biotechnol. Appl. Biochem. 13(3):412-21). To facilitate optimal folding of the IGF-II moiety, appropriate concentrations of reduced and oxidized glutathione are preferably added to the cellular milieu to promote disulfide bond formation. In the event that a fusion protein with disulfide bonds is incompletely soluble, any insoluble material is preferably treated with a chaotropic agent such as urea to solubilize denatured protein and refolded in a buffer having appropriate concentrations of reduced and oxidized glutathione, or other oxidizing and reducing agents, to facilitate formation of appropriate disulfide bonds (Smith *et al.* (1989) J. Biol. Chem. 264(16):9314-21). For example, IGF-I has been refolded using 6M guanidine-HCl and 0.1 M tris(2-carboxyethyl)phosphine reducing agent for denaturation and reduction of IGF-II (Yang *et al.* (1999) J. Biol. Chem. 274(53):37598-604). Refolding of proteins was accomplished in 0.1M Tris-HCl buffer (pH8.7) containing 1mM oxidized glutathione, 10 mM reduced glutathione, 0.2M KCl and 1mM EDTA.

In vivo expression

[0079] A nucleic acid encoding a therapeutic protein, preferably a secreted therapeutic protein, can be advantageously provided directly to a patient suffering from a disease, or may be provided to a cell *ex vivo*, followed by adminstration of the living cell to the patient. *In vivo* gene therapy methods known in the art include providing purified DNA (*e.g.* as in a plasmid) , providing the DNA in a viral vector, or providing the DNA in a liposome or other vesicle (see, for example, U.S. Patent No. 5,827,703, disclosing lipid

carriers for use in gene therapy, and U.S. Patent No. 6,281,010, providing adenoviral vectors useful in gene therapy).

[0080] Methods for treating disease by implanting a cell that has been modified to express a recombinant protein are also well known. See, for example, U.S. Patent No. 5,399,346, disclosing methods for introducing a nucleic acid into a primary human cell for introduction into a human. Although use of human cells for *ex vivo* therapy is preferred in some embodiments, other cells such as bacterial cells may be implanted in a patient's vasculature, continuously releasing a therapeutic agent. See, for example, U.S. Patent Nos. 4,309,776 and 5,704,910.

[0081] Methods of the invention are particularly useful for targeting a protein directly to a subcellular compartment without requiring a purification step. In one embodiment, an IGF-II fusion protein is expressed in a symbiotic or attenuated parasitic organism that is administered to a host. The expressed IGF-II fusion protein is secreted by the organism, taken up by host cells and targeted to their lysosomes.

[0082] In some embodiments of the invention, GILT proteins are delivered *in situ* via live *Leishmania* secreting the proteins into the lysosomes of infected macrophage. From this organelle, it leaves the cell and is taken up by adjacent cells not of the macrophage lineage. Thus, the GILT tag and the therapeutic agent necessarily remain intact while the protein resides in the macrophage lysosome. Accordingly,.when GILT proteins are expressed *in situ*, they are preferably modified to ensure compatibility with the lysosomal environment. Human ß-glucuronidase (human "GUS"), an exemplary therapeutic portion, normally undergoes a C-terminal peptide cleavage either in the lysosome or during transport to the lysosome (e.g. between residues 633 and 634 in GUS). Thus, in embodiments where a GUS-GILT construct is to be expressed by *Leishmania* in a macrophage lysosome human GUS is preferably modified to render the protein resistant to cleavage, or the residues following

residue 633 are preferably simply omitted from a GILT fusion protein. Similarly, IGF-II, an exemplary targeting portion, is preferably modified to increase its resistance to proteolysis, or a minimal binding peptide (e.g. as identified by phage display or yeast two hybrid) is substituted for the wildtype IGF-II moiety.

Administration

[0083] The targeted therapeutics discovered according to the present invention can be administered to a mammalian host by any route. Thus, as appropriate, administration can be oral or parenteral, including intravenous and intraperitoneal routes of administration. In addition, administration can be by periodic injections of a bolus of the therapeutic or can be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an i.v. bag). In certain embodiments, the therapeutics of the instant invention can be pharmaceutical-grade. That is, certain embodiments comply with standards of purity and quality control required for administration to humans. Veterinary applications are also within the intended meaning as used herein.

[0084] The formulations, both for veterinary and for human medical use, of the therapeutics according to the present invention typically include such therapeutics in association with a pharmaceutically acceptable carrier therefor and optionally other ingredient(s). The carrier(s) can be "acceptable" in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient thereof. Pharmaceutically acceptable carriers, in this regard, are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound,

use thereof in the compositions is contemplated. Supplementary active compounds (identified according to the invention and/or known in the art) also can be incorporated into the compositions. The formulations can conveniently be presented in dosage unit form and can be prepared by any of the methods well known in the art of pharmacy/microbiology. In general, some formulations are prepared by bringing the therapeutic into association with a liquid carrier or a finely divided solid carrier or both, and then, if necessary, shaping the product into the desired formulation.

[0085] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include oral or parenteral, e.g., intravenous, intradermal, inhalation, transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. Ph can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

[0086] Useful solutions for oral or parenteral administration can be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences, (Gennaro, A., ed.), Mack Pub., 1990. Formulations for parenteral administration also can include glycocholate for buccal administration, methoxysalicylate for rectal administration, or cutric acid for vaginal administration. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Suppositories for rectal administration also can be prepared by

mixing the drug with a non-irritating excipient such as cocoa butter, other glycerides, or other compositions that are solid at room temperature and liquid at body temperatures. Formulations also can include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration can include glycerol and other compositions of high viscosity. Other potentially useful parenteral carriers for these therapeutics include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration can contain as excipients, for example, lactose, or can be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Retention enemas also can be used for rectal delivery.

[0087] Formulations of the present invention suitable for oral administration can be in the form of discrete units such as capsules, gelatin capsules, sachets, tablets, troches, or lozenges, each containing a predetermined amount of the drug; in the form of a powder or granules; in the form of a solution or a suspension in an aqueous liquid or non-aqueous liquid; or in the form of an oil-in-water emulsion or a water-in-oil emulsion. The therapeutic can also be administered in the form of a bolus, electuary or paste. A tablet can be made by compressing or moulding the drug optionally with one or more accessory ingredients. Compressed tablets can be prepared by compressing, in a suitable machine, the drug in a free-flowing form such as a powder or granules, optionally mixed by a binder, lubricant, inert diluent, surface active or dispersing agent. Moulded tablets can be made by moulding, in a suitable machine, a mixture of the powdered drug and suitable carrier moistened with an inert liquid diluent.

[0088] Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients. Oral compositions prepared using a fluid carrier for use as a mouthwash include the compound in the fluid carrier and are applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose; a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0089] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition can be sterile and can be fluid to the extent that easy syringability exists. It can be stable under the conditions of manufacture and storage and can be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by

various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0090] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation include vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0091] Formulations suitable for intra-articular administration can be in the form of a sterile aqueous preparation of the therapeutic which can be in microcrystalline form, for example, in the form of an aqueous microcrystalline suspension. Liposomal formulations or biodegradable polymer systems can also be used to present the therapeutic for both intra-articular and ophthalmic administration.

[0092] Formulations suitable for topical administration, including eye treatment, include liquid or semi-liquid preparations such as liniments, lotions, gels, applicants, oil-in-water or water-in-oil emulsions such as creams, ointments or pasts; or solutions or suspensions such as drops. Formulations for topical administration to the skin surface can be prepared by dispersing the therapeutic with a dermatologically acceptable carrier such as a

lotion, cream, ointment or soap. In some embodiments, useful are carriers capable of forming a film or layer over the skin to localize application and inhibit removal. Where adhesion to a tissue surface is desired the composition can include the therapeutic dispersed in a fibrinogen-thrombin composition or other bioadhesive. The therapeutic then can be painted, sprayed or otherwise applied to the desired tissue surface. For topical administration to internal tissue surfaces, the agent can be dispersed in a liquid tissue adhesive or other substance known to enhance adsorption to a tissue surface. For example, hydroxypropylcellulose or fibrinogen/thrombin solutions can be used to advantage. Alternatively, tissue-coating solutions, such as pectin-containing formulations can be used.

[0093] For inhalation treatments, such as for asthma, inhalation of powder (self-propelling or spray formulations) dispensed with a spray can, a nebulizer, or an atomizer can be used. Such formulations can be in the form of a finely comminuted powder for pulmonary administration from a powder inhalation device or self-propelling powder-dispensing formulations. In the case of self-propelling solution and spray formulations, the effect can be achieved either by choice of a valve having the desired spray characteristics (i.e., being capable of producing a spray having the desired particle size) or by incorporating the active ingredient as a suspended powder in controlled particle size. For administration by inhalation, the therapeutics also can be delivered in the form of an aerosol spray from a pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Nasal drops also can be used.

[0094] Systemic administration also can be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants generally are known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and filsidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal

sprays or suppositories. For transdermal administration, the therapeutics typically are formulated into ointments, salves, gels, or creams as generally known in the art.

[0095] In one embodiment, the therapeutics are prepared with carriers that will protect against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials also can be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811. Microsomes and microparticles also can be used.

[0096] Oral or parenteral compositions can be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0097] Generally, the therapeutics identified according to the invention can be formulated for parenteral or oral administration to humans or other mammals, for example, in therapeutically effective amounts, e.g., amounts which provide appropriate concentrations of the drug to target tissue for a time sufficient to induce the desired effect. Additionally, the

therapeutics of the present invention can be administered alone or in combination with other molecules known to have a beneficial effect on the particular disease or indication of interest. By way of example only, useful cofactors include symptom-alleviating cofactors, including antiseptics, antibiotics, antiviral and antifungal agents and analgesics and anesthetics.

[0098] The effective concentration of the therapeutics identified according to the invention that is to be delivered in a therapeutic composition will vary depending upon a number of factors, including the final desired dosage of the drug to be administered and the route of administration. The preferred dosage to be administered also is likely to depend on such variables as the type and extent of disease or indication to be treated, the overall health status of the particular patient, the relative biological efficacy of the therapeutic delivered, the formulation of the therapeutic, the presence and types of excipients in the formulation, and the route of administration. In some embodiments, the therapeutics of this invention can be provided to an individual using typical dose units deduced from the earlier-described mammalian studies using non-human primates and rodents. As described above, a dosage unit refers to a unitary, i.e. a single dose which is capable of being administered to a patient, and which can be readily handled and packed, remaining as a physically and biologically stable unit dose comprising either the therapeutic as such or a mixture of it with solid or liquid pharmaceutical diluents or carriers.

[0099] In certain embodiments, organisms are engineered to produce the therapeutics identified according to the invention. These organisms can release the therapeutic for harvesting or can be introduced directly to a patient. In another series of embodiments, cells can be utilized to serve as a carrier of the therapeutics identified according to the invention.

[0100] Therapeutics of the invention also include the "prodrug" derivatives. The term prodrug refers to a pharmacologically inactive (or partially inactive) derivative of a

parent molecule that requires biotransformation, either spontaneous or enzymatic, within the organism to release or activate the active component. Prodrugs are variations or derivatives of the therapeutics of the invention which have groups cleavable under metabolic conditions. Prodrugs become the therapeutics of the invention which are pharmaceutically active *in vivo*, when they undergo solvolysis under physiological conditions or undergo enzymatic degradation. Prodrug of this invention can be called single, double, triple, and so on, depending on the number of biotransformation steps required to release or activate the active drug component within the organism, and indicating the number of functionalities present in a precursor-type form. Prodrug forms often offer advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985 and Silverman, The Organic Chemistry of Drug Design and Drug Action, pp. 352-401, Academic Press, San Diego, Calif., 1992). Moreover, the prodrug derivatives according to this invention can be combined with other features to enhance bioavailability.

Examples

<u>Example 1. GILT constructs</u>

[0101] IGF-II cassettes have been synthesized by ligation of a series of overlapping oligos and cloned into Pir1-SAT, a standard *Leishmania* expression vector. 4 IGF-II cassettes have been made: one that encodes the wildtype mature polypeptide, one with a Δ1-7 deletion, one with a Y27L mutation, and one with both mutations. These mutations are reported to reduce binding of IGF-II to the other receptors while not affecting binding to the M6P receptor.

[0102] The coding sequence of human IGF-II is shown in Figure 1. The protein is synthesized as a pre-pro-protein with a 24 amino acid signal peptide at the amino terminus

and a 89 amino acid carboxy terminal region both of which are removed post-translationally, reviewed in (O'Dell *et al.* (1998) Int. J. Biochem Cell Biol. 30(7):767-71. The mature protein is 67 amino acids. A *Leishmania* codon optimized version of the mature IGF-II is shown in Figure 2 (Langford *et al.* (1992) Exp. Parasitol 74(3):360-1). This cassette was constructed by annealing overlapping oligonucleotides whose sequences are shown in Table 2. Additional cassettes containing a deletion of amino acids 1-7 of the mature polypeptide (Δ1-7), alteration of residue 27 from tyrosine to leucine (Y27L) or both mutations (Δ1-7,Y27L) were made to produce IGF-II cassettes with specificity for only the desired receptor as described below. To make the wildtype IGF-II cassette, oligos GILT1-9 were annealed and ligated. To make the Y27L cassette, oligos 1, 12, 3, 4, 5, 16, 7, 8 and 9 were annealed and ligated. After ligation, the two cassettes were column purified. Wildtype and Y27L cassettes were amplified by PCR using oligos GILT 20 and 10 and the appropriate template. To incorporate the Δ1-7 deletion, the two templates were amplified using oligos GILT 11 and 10. The resulting 4 IGF-II cassettes (wildtype, Y27L, Δ1-7, and Y27LΔ1-7) were column purified, digested with XbaI, gel purified and ligated to XbaI cut Pir1-SAT.

[0103] Gene cassettes were then cloned between the XmaI site (not shown) upstream of XbaI in the vector and the AscI site in such a way as to preserve the reading frame. An overlapping DAM methylase site at the 3' XbaI site permitted use of the 5' XbaI site instead of the XmaI site for cloning. The AscI site adds a bridge of 3 amino acid residues.

TABLE 3. Oligonucleotides used in the construction of Pir-GILT vectors.

NAME	SEQUENCE	POSITION
GILT 1	GCGGCGGCGAGCTGGTGGACACGCTGCAGTTCGTGTGCGGCGACCGCGGC	48-97 top strand
GILT 2	TTCTACTTCAGCCGCCCGGCCAGCCGCGTGAGCCGCCGCAGCCGCGGCAT	98-147 top strand
GILT 3	CGTGGAGGAGTGCTGCTTCCGCAGCTGCGACCTGGCGCTGCTGGAGACGT	148-197 top strand
GILT 4	ACTGCGCGACGCCGGCGAAGTCGGAGTAAGATCTAGAGCG	198-237 top strand
GILT 5	AGCGTGTCCACCAGCTCGCCGCCGCACAGCGTCTCGCTCGGGCGGTACGC	72-23 bottom
GILT 6	GGCTGGCCGGGCGGCTGAAGTAGAAGCCGCGGTCGCCGCACACGAACTGC	122-73 bottom
GILT 7	GCTGCGGAAGCAGCACTCCTCCACGATGCCGCGGCTGCGGCGGCTCACGC	172-123 bottom
GILT 8	CTCCGACTTCGCCGGCGTCGCGCAGTACGTCTCCAGCAGCGCCAGGTCGCA	223-173 bottom
GILT 9	CCGTCTAGAGCTCGGCGCGCCGGCGTACCGCCCGAGCGAGACGCTGT	1-47 top strand
GILT 10	CGCTCTAGATCTTACTCCGACTTCG	237-202 bottom
GILT 11	CCGTCTAGAGCTCGGCGCGCCGCTGTGCGGCGGCGAGCTGGTGGAC	1-67, Δ23-43 top
GILT 12	TTCCTGTTCAGCCGCCCGGCCAGCCGCGTGAGCCGCCGCAGCCGCGGCAT	98-147 (Y27L) top
GILT 16	GGCTGGCCGGGCGGCTGAACAGGAAGCCGCGGTCGCCGCACACGAACTGC	122-73 (Y27L) bot
GILT 20	CCGTCTAGAGCTCGGCGCGCCGGCG	1-25 top strand

[0104] The purpose of incorporating the indicated mutations into the IGF-II cassette is to insure that the fusion proteins are targeted to the appropriate receptor. Human IGF-II has a high degree of sequence and structural similarity to IGF-I (see, for example Figure 6) and the B and A chains of insulin (Terasawa *et al.* (1994) Embo J. 13(23):5590-7). Consequently, it is not surprising that these hormones have overlapping receptor binding specificities. IGF-II binds to the insulin receptor, the IGF-I receptor and the cation independent mannose 6-phosphate/IGF-II receptor (CIM6P/IGF-II). The CIM6P/IGF-II receptor is a dual activity receptor acting as a receptor for IGF-II and as a mannose 6-phosphate receptor involved in sorting of lysosomal hydrolases. For a number of years, these two activities were attributed to separate proteins until it was determined that both activities resided in a single protein (Morgan *et al.* (1987) Nature 329(6137):301-7); (Tong *et al.* (1988) J. Biol. Chem. 263(6):2585-8).

[0105] The most profound biological effects of IGF-II, such as its mitogenic effect, are mediated through the IGF-I receptor rather than the CIM6P/IGF-II receptor, reviewed in (Ludwig *et al.* (1995) Trends in Cell Biology 5:202-206) also see (Korner *et al.* (1995) J. Biol. Chem. 270(1):287-95). It is thought that the primary result of IGF-II binding to the CIM6P/IGF-II receptor is transport to the lysosome for subsequent degradation. This represents an important means of controlling IGF-II levels and explains why mice carrying

null mutants of the CIM6P/IGF-II receptor exhibit perinatal lethality unless IGF-II is also deleted (Lau *et al.* (1994) Genes Dev. 8(24):2953-63); (Wang *et al.* (1994) Nature 372(6505):464-7); (Ludwig *et al.* (1996) Dev. Biol. 177(2):517-35). In methods of the present invention, it is desirable to have the IGF-II fusion proteins bind to the CIM6P/IGF-II receptor. The Y27L and Δ1-7 mutations reduce IGF-II binding to the IGF-I and insulin receptors without altering the affinity for the CIM6P/IGF-II receptor (Sakano *et al.* (1991) J. Biol. Chem. 266(31):20626-35); (Hashimoto *et al.* (1995) J. Biol. Chem. 270(30):18013-8). Therefore, according to the invention, these mutant forms of IGF-II should provide a means of targeting fusion proteins specifically to the CIM6P/IGF-II receptor.

[0106] In one experiment, 4 different IGF-II cassettes with the appropriate sequences, wild type, Δ1-7, Y27L and Δ1-7/Y27L are made. ß-GUS cassettes are fused to IGF-II cassettes and these constructs are put into parasites. Alpha-galactosidase cassettes are also fused to the IGF-II cassettes. GUS fusions have been tested and shown to produce enzymatically active protein.

[0107] One preferred construct, shown in Figure 3, includes the signal peptide of the *L. mexicana* secreted acid phosphatase, SAP-1, cloned into the XbaI site of a modified Pir1-SAT in which the single SalI site has been removed. Fused in-frame is the mature *β*-GUS sequence, connected to an IGF-II tag by a bridge of three amino acids.

Example 2. GILT protein preparation

[0108] *L. mexicana* expressing and secreting ß-GUS were grown at 26°C in 100 ml Standard Promastigote medium (M199 with 40 mM HEPES, pH 7.5, 0.1 mM adenine, 0.0005% hemin, 0.0001% biotin, 5% fetal bovine serum, 5% embryonic fluid, 50 units/ml penicillin, 50 µg/ml streptomycin and 50µg/ml nourseothricin). After reaching a density of approximately 5 x 10^6 promastigotes/ml, the promastigotes were collected by centrifugation

for 10 min. at 1000 x g at room temperature; these promastigotes were used to inoculate 1 liter of low protein medium (M199 supplemented with 0.1 mM adenine, 0.0001% biotin, 50 units/ml penicillin and 50 μg/ml streptomycin) at room temperature. The 1 liter cultures were contained in 2 liter capped flasks with a sterile stir bar so that the cultures could be incubated at 26°C with gentle stirring. The 1 liter cultures were aerated twice a day by moving them into a laminar flow hood, removing the caps and swirling vigorously before replacing the caps. When the cultures reached a density of 2-3 x 10^7 promastigotes/ml, the cultures were centrifuged as described above except the promastigote pellet was discarded and the media decanted into sterile flasks. The addition of 434 g $(NH_4)_2SO_4$ per liter precipitated active GUS protein from the medium; the salted out medium was stored at 4°C overnight. Precipitated proteins were harvested either by centrifugation at 10,500 x g for 30 min. or filtration through Gelman Supor-800 membrane; the proteins were resuspended in 10 mM Tris pH 8, 1 mM $CaCl_2$ and stored at –80°C until dialysis. The crude preparations from several liters of medium were thawed, pooled, placed in dialysis tubing (Spectra/Por –7, MWCO 25,000), and dialyzed overnight against two 1 liter volumes of DMEM with bicarbonate (Dulbecco's Modified Eagle's Medium).

Example 3. GILT uptake assay

[0109] Skin fibroblast line GM4668 (human, NIGMS Human Genetic Mutant Cell Repository) is derived from a patient with mucopolysaccharidosis VII; the cells therefore have little or no *β*-GUS activity. GM4668 cells are therefore particularly useful for testing the uptake of GUS-GILT constructs into human cells. GM4668 cells were cultured in 12-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) fetal calf serum at 37°C in 5% CO_2. Fibroblasts were cultured overnight in the presence of about 150 units of preparations of *Leishmania*-expressed human ß-

glucuronidase (GUS), GUS-IGF-II fusion protein (GUS-GILT), or mutant GUS-IGF-II fusion protein (GUSΔ-GILT) prepared as described in Example 2. Control wells contained no added enzyme (DMEM media blank). After incubation, media was removed from the wells and assayed in triplicate for GUS activity. Wells were washed five times with 1 ml of 37^{0}C phosphate-buffered saline, then incubated for 15 minutes at room temperature in 0.2 ml of lysis buffer (10 mM Tris, pH7.5, 100 mM NaCl, 5 mM EDTA, 2mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma), and 1% NP-40). Cell lysates were transferred to microfuge tubes, then spun at 13,000 rpm for 5 minutes to remove cell debris. Three 10 µL aliquots of lysate were assayed for protein concentration (Pierce Micro BCA protein assay, Pierce, IL).

[0110] Three 38 µL aliquots of lysate were assayed for GUS activity using a standard fluorometric assay adapted from (Wolfe *et al.* (1996) Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders 263-274). Assays are done in disposable fluorimeter cuvettes. 150 µl of reaction mix is added to each cuvette. 1 ml reaction mix is 860 µl H2O, 100µl 1M NaAcetate, 40µl 25X ß-GUS substrate mix. (25X ß-GUS substrate mix is a suspension of 250 mg 4-methylumbelliferyl-ß-D glucuronide in 4.55 ml ethanol stored at –20°C in a dessicator. 38µl of sample are added to the reaction mix and the reaction is incubated at 37 °C . Reactions are terminated by addition of 2 ml stop solution (10.6 g Na_2CO_3, 12.01 g glycine, H2O to 500 ml, pH 10.5). Fluorescence output is then measured by fluorimeter.

[0111] Results of the uptake experiment indicate that the amount of cell-associated GUS-GILT is 10-fold greater that that of the unmodified GUS (Figure 4). The double mutant construct is about 5-fold more effective than unmodified GUS. These results indicate that the GILT technology is an effective means of targeting a lysosomal enzyme for uptake. Uptake can also be verified using standard immunofluorescence techniques.

Example 4. Competition experiments

[0112] To verify that the GILT –mediated uptake occurs via the IGF-II binding site on the cation-independent M6P receptor, competition experiments were performed using recombinant IGF-II. The experimental design was identical to that described above except that GM4668 fibroblasts were incubated with indicated proteins in DMEM minus serum +2%BSA for about 18 hours. Each ß-GUS derivative was added at 150 U per well. 2.85 μg IGF-II was added to each well for competition. This represents approximately a 100 fold molar excess over GILT-GUS, a concentration sufficient to compete for binding to the M6P/IGF-II receptor.

[0113] Results of the competition experiment are depicted in Figure 5. In the absence of IGF-II over 24 units of GILT-GUS/ mg lysate were detected. Upon addition of IGF-II, the amount of cell associated GILT-GUS fell to 5.4 U. This level is similar to the level of unmodified GUS taken up by the fibroblasts. Thus, the bulk of the GILT protein uptake can be competed by IGF-II indicating that the uptake is indeed occurring through a specific receptor-ligand interaction.

Example 5. Gene Product Expression in serum free media

[0114] Expression products can also be isolated from serum free media. In general, the expression strain is grown in medium with serum, diluted into serum free medium, and allowed to grow for several generations, preferably 2-5 generations, before the expression product is isolated. For example, production of secreted targeted therapeutic proteins can be isolated from *Leishmania mexicana* promastigotes that are cultured initially in 50 ml 1X M199 medium in a 75 cm2 flask at 27° C. When the cell density reaches 1-3x 10^7/ml, the culture is used to inoculate 1.2 L of M199 media. When the density of this

culture reaches about $5x10^6$/ml, the cells are harvested by centrifugation, resuspended in 180 ml of the supernatant and used to inoculate 12 L of "Zima" medium in a 16 L spinner flask. The initial cell density of this culture is typically about $5x\ 10^5$ /ml. This culture is expanded to a cell density of about 1.0 – 1.7 x $10e^7$ cells/ml. When this cell density is reached, the cells are separated from the culture medium by centrifugation and the supernatant is filtered at 4°C through a 0.2 μ filter to remove residual promastigotes. The filtered media was concentrated from 12.0 L to 500 ml using a tangential flow filtration device (MILLIPORE Prep/Scale-TFF cartridge).

[0115] Preferred growth media for this method are M199 and "Zima" growth media. However, other serum containing and serum free media are also useful. M199 growth media is as follows: (1L batch) = 200 ml 5X M199 (with phenol red pH indicator) + 637 ml H_2O, 50.0 ml FBS, 50.0 ml EF, 20.0 ml of 50 micrograms/ml SAT, 2.0 ml of 0.25% hemin in 50% triethanolamine , 10 ml of 10mM adenine in 50mM Hepes pH 7.5, 40.0 ml of 1M Hepes pH 7.5, 1ml of 0.1% biotin in 95% ethanol, 10.0 ml of penicillin/streptomycin. All sera used are inactivated by heat. The final volume = 1 L and is filter sterilized. "Zima" modified M199 media is as follows: (20.0 L batch) = 217.8g M199 powder (–)phenol red + 7.0g sodium bicarbonate, 200.0 ml of 10mM adenine in 50mM Hepes pH 7.5, 800.0 ml 0f Hepes free acid pH 7.5, 20.0 ml 0.1% biotin in 95% ethanol, 200.0 ml penicillin/streptomycin, Final volume = 20.0 L and is filter sterilized.

[0116] The targeted therapeutic proteins are preferably purified by Concanavalin A (ConA) chromatography. For example, when a culture reaches a density of > 1.0 x 10^7 promastigotes/ml, *L. mexicana* are removed by centrifugation, 10 min at 500 x g. The harvested culture medium is passed through a 0.2 μm filter to remove particulates before being loaded directly onto a ConA-agarose column (4% cross-linked beaded agarose, Sigma). The ConA-agarose column is pretreated with 1 M NaCl, 20 mM Tris pH 7.4, 5 mM each of

$CaCl_2$, $MgCl_2$ and $MnCl_2$ and then equilibrated with 5 volumes of column buffer (20 mM Tris pH 7.4, 1 mM $CaCl_2$, and 1 mM $MnCl_2$). A total of 179,800 units (nmol/hr) of GUS activity (in 2 L) in culture medium is loaded onto a 22 ml ConA agarose column. No activity is detectable in the flow through or wash. The GUS activity is eluted with column buffer containing 200 mM methyl mannopyranoside. Eluted fractions containing the activity peak are pooled and concentrated. Uptake and competition experiments were performed as described in Examples 3 and 4, except that the organisms were grown in serum-free medium and purified with ConA; about 350-600 units of enzyme were applied to the fibroblasts. Results are shown in Figure 7.

Example 6. Competition experiments using denatured IGF-II as competitor

[0117] The experiment in Example 4 is repeated using either normal or denatured IGF-II as competitor. As in Example 4, the amount of cell-associated GUS-GILT is reduced when coincubated with normal IGF-II concentrations that are effective for competition but, at comparable concentrations, denatured IGF-II has little or no effect.

Example 7. Binding uptake and halflife experiments

[0118] Binding of GUS-GILT proteins to the M6P/IGF-II receptor on fibroblasts are measured and the rate of uptake is assessed similar to published methods (York *et al.* (1999) J. Biol. Chem. 274(2):1164-71). GM4668 fibroblasts cultured in 12 well culture dishes as described above are washed in ice-cold media minus serum containing 1% BSA. Ligand, (either GUS, GUS-GILT or GUS-ΔGILT, or control proteins) is added to cells in cold media minus serum plus 1% BSA. Upon addition of ligand, the plates are incubated on ice for 30 minutes. After 30 minutes, ligand is removed and cells are washed quickly 5 times with ice cold media. Wells for the 0 time point receive 1 ml ice cold stripping buffer (0.2 M

Acetic acid, pH 3.5, 0.5M NaCl). The plate is then floated in a 37° water bath and 0.5 ml prewarmed media is added to initiate uptake. At every stopping point, 1 ml of stripping buffer is added. When the experiment is over, aliquots of the stripping buffer are saved for fluorometric assay of ß-glucuronidase activity as described in Example 3. Cells are then lysed as described above and the lysate assayed for ß-glucuronidase activity.

[0119] It is expected that GUS-GILT is rapidly taken up by fibroblasts in a matter of minutes once the temperature is shifted to 37°C (York *et al.* (1999) J. Biol. Chem. 274(2):1164-71) and that the enzyme activity persists in the cells for many hours.

Example 8. *In vivo* therapy

[0120] GUS minus mice generated by heterozygous matings of B6.C-H-2^{bml}/ByBIR-gus^{mps}/+ mice (Birkenmeier *et al.* (1989) J. Clin. Invest 83(4):1258-6) are used to assess the effectiveness of GUS-GILT or derivatives in enzyme replacement therapy. Two formats are used. In one format, 3-4 animals are given a single injection of 20,000U of enzyme in 100 µl enzyme dilution buffer (150 mM NaCl, 10 mM Tris, pH7.5). Mice are killed 72-96 hours later to assess the efficacy of the therapy. In a second format, mice are given weekly injections of 20,000 units over 3-4 weeks and are killed 1 week after the final injection. Histochemical and histopathologic analysis of liver, spleen and brain are carried out by published methods (Birkenmeier *et al.* (1991) Blood 78(11):3081-92); (Sands *et al.* (1994) J. Clin. Invest 93(6):2324-31); (Daly *et al.* (1999) Proc. Natl. Acad. Sci. USA 96(5):2296-300). In the absence of therapy, cells (*e.g.* macrophages and Kupffer cells) of GUS minus mice develop large intracellular storage compartments resulting from the buildup of waste products in the lysosomes. It is anticipated that in cells in mice treated with GUS-GILT constructs, the size of these compartments will be visibly reduced or the compartments will shrink until they are no longer visible with a light microscope.

[0121] Similarly, humans with lysosomal storage diseases will be treated using constructs targeting an appropriate therapeutic portion to their lysosomes. In some instances, treatment will take the form of regular (*e.g.* weekly) injections of a GILT protein. In other instances, treatment will be achieved through administration of a nucleic acid to permit persistent *in vivo* expression of a GILT protein, or through administration of a cell (*e.g.* a human cell, or a unicellular organism) expressing the GILT protein in the patient. For example, the GILT protein can be expressed *in situ* using a *Leishmania* vector as described in U.S. Patent No. 6,020,144, issued February 1, 2000; U.S. Provisional Application No. 60/250,446; and U.S. Provisional Application Attorney Docket No. SYM-005PRA, "Protozoan Expression Systems for Lysosomal Storage Disease Genes", filed May 11, 2001.

Example 9.

[0122] The objective of these experiments is to evaluate the efficacy of GILT-modified alpha-galactosidase A (α-GAL A) as an enzyme replacement therapy for Fabry's disease.

[0123] Fabry's disease is a lysosomal storage disease resulting from insufficient activity of α-GAL A, the enzyme responsible for removing the terminal galactose from GL-3 and other neutral sphingolipids. The diminished enzymatic activity occurs due to a variety of missense and nonsense mutations in the x-linked gene. Accumulation of GL-3 is most prevalent in lysosomes of vascular endothelial cells of the heart, liver, kidneys, skin and brain but also occurs in other cells and tissues. GL-3 buildup in the vascular endothelial cells ultimately leads to heart disease and kidney failure.

[0124] Enzyme replacement therapy is an effective treatment for Fabry's disease, and its success depends on the ability of the therapeutic enzyme to be taken up by the lysosomes of cells in which GL-3 accumulates. The Genzyme product, Fabrazyme, is

recombinant α-GAL A produced in DUKX B11 CHO cells that has been approved for treatment of Fabry's patients in Europe due to its demonstrated efficacy.

[0125] The ability of Fabrazyme to be taken up by cells and transported to the lysosome is due to the presence of mannose 6-phosphate (M6P) on its N-linked carbohydrate. Fabrazyme is delivered to lysosomes through binding to the mannose-6-phosphate/IGF-II receptor (M6P/IGF-Iir), present on the cell surface of most cell types, and subsequent receptor mediated endocytosis. Fabrazyme reportedly has three N-linked glycosylation sites at ASN residues 108, 161, and 184. The predominant carbohydrates at these positions are fucosylated biantennary bisialylated complex, monophosphorylated mannose-7 oligomannose, and biphosphorylated mannose-7 oligomannose, respectively.

[0126] The glycosylation independent lysosomal targeting (GILT) technology of the present invention directly targets therapeutic proteins to the lysosome via a different interaction with the M6P/IGF-Iir. A targeting ligand is derived from mature human IGF-II, which also binds with high affinity to the M6P/IGF-Iir. In current applications, the IGF-II tag is provided as a c-terminal fusion to the therapeutic protein, although other configurations are feasible including cross-linking. The competency of GILT-modified enzymes for uptake into cells has been established using GILT-modified ß-glucuronidase, which is efficiently taken up by fibroblasts in a process that is competed with excess IGF-II. Advantages of the GILT modification are increased binding to the M6P/IGF-II receptor, enhanced uptake into lysosomes of target cells, altered or improved pharmacokinetics, and expanded, altered or improved range of tissue distribution. The improved range of tissue distributions could include delivery of GILT-modified α-GAL A across the blood-brain barrier since IGF proteins demonstrably cross the blood-brain barrier.

[0127] Another advantage of the GILT system is the ability to produce uptake-competent proteins in non-mammalian expression systems where M6P modifications do not

occur. In certain embodiments, GILT-modified protein will be produced primarily in CHO cells. In certain others, the GILT tag will be placed at the c-terminus of α-GAL A although the invention is not so limited.

INCORPORATION BY REFERENCE

[0128] The disclosure of each of the patent documents, scientific publications, and Protein Data Bank records disclosed herein, and U.S. Provisional Application No. 60/250,446, filed November 30, 2000; U.S. Provisional Application 60/287,531, filed April 30, 2001; U.S. Provisional Application 60/290,281, filed May 11, 2001; U.S. Provisional Application 60/304,609, filed July 10. 2001; U.S. Provisional Application No. 60/329,461, filed October 15, 2001; International Patent Application Serial No. PCT/US01/44935, filed November 30, 2001; U.S. Provisional Application No. 60/351,276, filed January 23, 2002; and U.S. Serial No. 10/______ (Attorney Docket No. SYM-008), filed April 30, 2002, are incorporated by reference into this application in their entirety.

[0129] What is claimed is:

CLAIMS

1. A targeted therapeutic comprising:
a therapeutic agent that is therapeutically active in a mammalian lysosome; and
means for binding an extracellular domain of human cation-independent mannose-6-phosphate receptor in a mannose-6-phosphate-independent manner.

2. The targeted therapeutic of claim 1, wherein the means for binding comprises retinoic acid or a derivative thereof.

3. The targeted therapeutic of claim 1, wherein the means for binding comprises a protein having an amino acid sequence at least 70% identical to a domain of urokinase-type plasminogen activator receptor.

4. The targeted therapeutic of claim 1, wherein the means for binding comprises IGF-II or an antibody variable domain.

5. The targeted therapeutic of claim 1, wherein association of the therapeutic agent with the means for binding is destabilized by a pH change from about pH 7.4 to about pH 5.5.

6. The targeted therapeutic of claim 1, wherein the means for binding binds to the extracellular domain with a submicromolar dissociation constant at about pH 7.4.

7. The targeted therapeutic of claim 6, wherein the dissociation constant is less than 10^{-8} M.

8. The targeted therapeutic of claim 6, wherein the dissociation constant is less than 10^{-9} M.

9. The targeted therapeutic of claim 6, wherein the dissociation constant is less than 10^{-10} M.

10. The targeted therapeutic of claim 6, wherein the dissociation constant is between 10^{-7} M and 10^{-11} M.

11. The targeted therapeutic of claim 6, wherein the means for binding binds to the extracellular domain with an dissociation constant at about pH 5.5 at least ten times the dissociation constant at about pH 7.4.

12. The targeted therapeutic of claim 11, wherein the dissociation constant at about pH 5.5 is at least 10^{-6} M.

13. A targeted therapeutic comprising:
a therapeutic agent that is therapeutically active in a mammalian lysosome; and
an unglycosylated lysosomal targeting domain that binds an extracellular domain of human cation-independent mannose-6-phosphate receptor.

14. A targeted therapeutic comprising:
a therapeutic agent that is therapeutically active in a human lysosome; and
a lysosomal targeting domain that binds an extracellular domain of human cation-independent mannose-6-phosphate receptor and
(i) does not bind a mutein in which amino acid 1572 of the human cation-independent mannose-6-phosphate receptor is changed from isoleucine to threonine; or
(ii) binds the mutein with a dissociation constant at least ten times the dissociation constant for binding the extracellular domain of human cation-independent mannose-6-phosphate receptor.

15. A targeted therapeutic comprising:
a therapeutic agent that is therapeutically active in a human lysosome; and
a lysosomal targeting domain that is capable of binding a receptor domain consisting essentially of repeats 10-15 of the human cation-independent mannose-6-phosphate receptor.

16. The targeted therapeutic of claim 15, wherein the lysosomal targeting domain is capable of binding a receptor domain consisting essentially of repeats 10-13 of the human cation-independent mannose-6-phosphate receptor.

17. The targeted therapeutic of claim 16, wherein the lysosomal targeting domain binds a receptor domain consisting essentially of repeats 11-12 of the human cation-independent mannose-6-phosphate receptor.

18. The targeted therapeutic of claim 17, wherein the lysosomal targeting domain binds a receptor domain consisting essentially of repeat 11 of the human cation-independent mannose-6-phosphate receptor.

19. The targeted therapeutic of claim 18, wherein the lysosomal targeting domain binds a receptor domain consisting essentially of amino acids 1508-1566 of the human cation-independent mannose-6-phosphate receptor.

20. The targeted therapeutic of claim 15, wherein the lysosomal targeting domain binds the receptor domain with a submicromolar dissociation constant at pH 7.4.

21. The targeted therapeutic of claim 20, wherein the dissociation constant is about 10^{-7} M.

22. The targeted therapeutic of claim 20, wherein the dissociation constant is less than about 10^{-7} M.

23. A targeted therapeutic comprising:

a therapeutic agent that is therapeutically active in a human lysosome; and

a binding moiety sufficiently duplicative of human IGF-II such that the binding moiety binds the human cation-independent mannose-6-phosphate receptor.

24. The targeted therapeutic of claim 23, wherein the binding moiety is an organic molecule having a three-dimensional shape representative of at least a portion of IGF-II.

25. The targeted therapeutic of claim 24, wherein the portion of IGF-II comprises amino acids 48-55 of human IGF-II.

26. The targeted therapeutic of claim 24, wherein the portion of IGF-II comprises at least three amino acids selected from the group consisting of amino acids 8, 48, 49, 50, 54, and 55 of human IGF-II.

27. The targeted therapeutic of claim 24, wherein the organic molecule has a hydrophobic moiety at a position representative of amino acid 48 of human IGF-II and has a positive charge at about pH 7.4 at a position representative of amino acid 49 of human IGF-II.

28. The targeted therapeutic of claim 23, wherein the binding moiety comprises a polypeptide comprising the amino acid sequence of IGF-I or of a mutein of IGF-I in which

(i) amino acids 55 and 56 are changed,

(ii) amino acids 1-4 are deleted or changed, or

(iii) amino acids 55 and 56 are changed and amino acids 1-4 are deleted or changed.

29. The targeted therapeutic of claim 23, wherein the binding moiety comprises a polypeptide comprising an amino acid sequence at least 60% identical to human IGF-II.

30. The targeted therapeutic of claim 29, wherein the amino acid sequence comprises, at positions corresponding to positions 54 and 55 of human IGF-II, amino acids each of which are uncharged or negatively charged at pH 7.4.

31. The targeted therapeutic of claim 23 wherein the binding moiety comprises a polypeptide having antiparallel alpha-helices separated by not more than five amino acids.

32. A targeted therapeutic comprising:

a therapeutic agent that is therapeutically active in a human lysosome; and

a polypeptide comprising the amino acid sequence phenylalanine-arginine-serine.

33. A targeted therapeutic comprising:

a therapeutic agent that is therapeutically active in a human lysosome; and

a polypeptide comprising an amino acid sequence at least 75% homologous to amino acids 48-55 of human IGF-II.

34. A targeted therapeutic comprising:

a therapeutic agent that is therapeutically active in a human lysosome;

amino acids 8-28 of human IGF-II; and

amino acids 41-61 of human IGF-II.

35. The targeted therapeutic of claim 34, wherein amino acids 8-28 and 41-61 are present in a single polypeptide.

36. A targeted therapeutic comprising:

a therapeutic agent that is therapeutically active in a human lysosome;

amino acids 41-61 of human IGF-II; and

a mutein of amino acids 8-28 of human IGF-II, the mutein differing from human IGF-II at a position selected from the group consisting of amino acid 9, amino acid 19, amino acid 26, and amino acid 27.

37. A therapeutic fusion protein comprising:

a therapeutic domain and

a subcellular targeting domain that binds to an extracellular domain of a receptor on an exterior surface of a cell and, upon internalization of the receptor, permits localization of the therapeutic domain to a subcellular compartment where the therapeutic domain is therapeutically active.

38. The therapeutic fusion protein of claim 37, wherein the subcellular compartment is selected from the group consisting of a lysosome, an endosome, endoplasmic reticulum, and golgi complex.

39. The therapeutic fusion protein of claim 38, wherein the subcellular compartment is a lysosome.

40. The therapeutic fusion protein of claim 37, wherein the receptor undergoes continuous endocytosis.

41. The therapeutic fusion protein of claim 37, wherein the therapeutic domain has a therapeutic enzymatic activity.

42. The therapeutic fusion protein of claim 41, wherein a cellular or subcellular deficiency in the enzymatic activity is associated with a human disease.

43. The therapeutic fusion protein of claim 42, wherein the human disease is a lysosomal storage disease.

44. A nucleic acid encoding the therapeutic fusion protein of claim 37.

45. A cell comprising the nucleic acid of claim 44.

46. A method of producing a therapeutic fusion protein, the method comprising the step of providing to the cell of claim 45 conditions permitting expression of the therapeutic fusion protein.

47 The method of claim 46, comprising culturing the cell *in vitro*.

48. The method of claim 46, comprising maintaining the cell inside a mammalian body.

49. The method of claim 46, further comprising harvesting the therapeutic fusion protein.

50. A method of treating a patient, the method comprising administering to the patient the therapeutic fusion protein of claim 37.

51. A method of treating a patient, the method comprising administering to the patient the nucleic acid of claim 44.

52. A method of treating a patient, the method comprising administering to the patient the cell of claim 45.

53. A method of treating a patient, the method comprising:

(i) synthesizing a targeted therapeutic comprising a therapeutic agent that is therapeutically active in a mammalian lysosome and a targeting moiety that binds human cation-independent mannose-6-phosphate receptor in a mannose-6-phosphate-independent manner; and

(ii) administering the targeted therapeutic to the patient.

54. The method of claim 53, further comprising, prior to step (i),

identifying a targeting moiety that binds human cation-independent mannose-6-phosphate receptor in a mannose-6-phosphate-independent manner.

55. The method of claim 54, wherein the targeting moiety is identified by screening a nucleic acid or peptide library.

56. A method of producing a targeted therapeutic, the method comprising the steps of:

(a) providing a molecular model defining a three-dimensional shape representative of at least a portion of human IGF-II;

(b) identifying a candidate IGF-II analog having a three-dimensional shape corresponding to the three-dimensional shape representative of at least a portion of human IGF-II; and

(c) producing a therapeutic agent directly or indirectly bound to the candidate IGF-II analog, wherein the therapeutic agent is therapeutically active in a mammalian lysosome.

57. The method of claim 56, further comprising determining whether the compound produced in step c binds to the human cation-independent mannose-6-phosphate receptor.

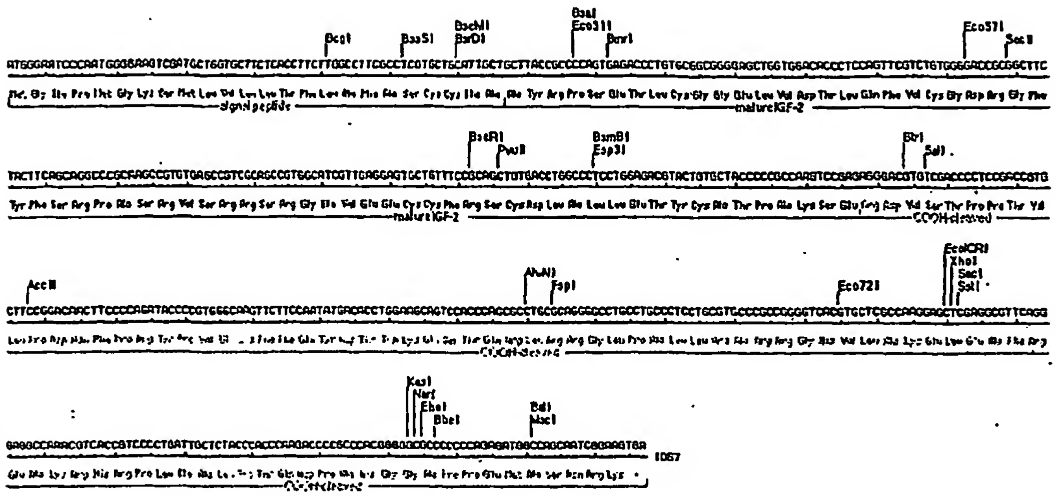


Figure 1.

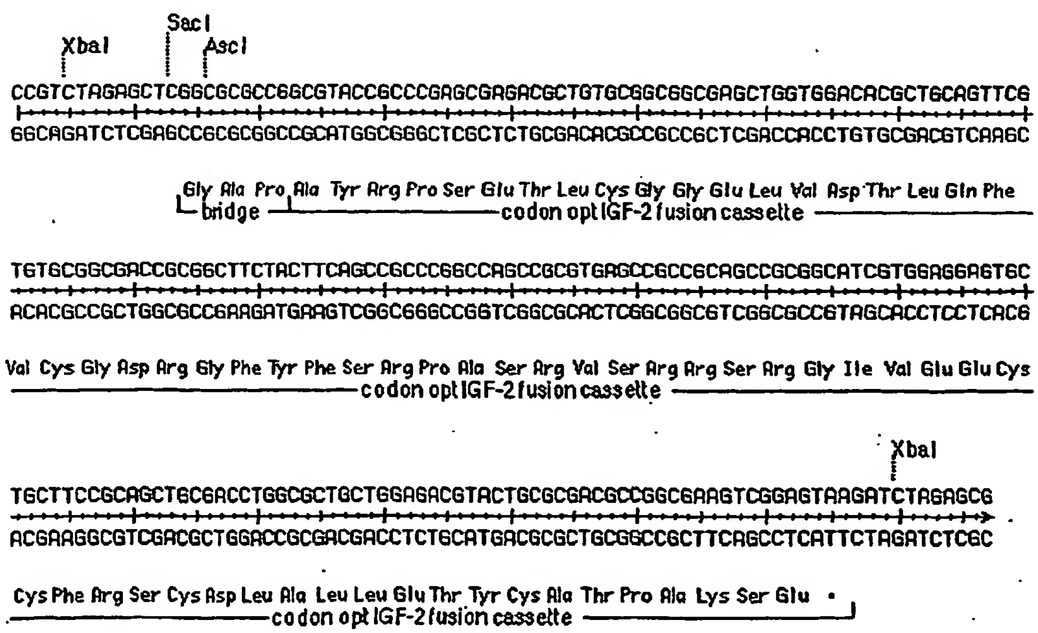


Figure 2.

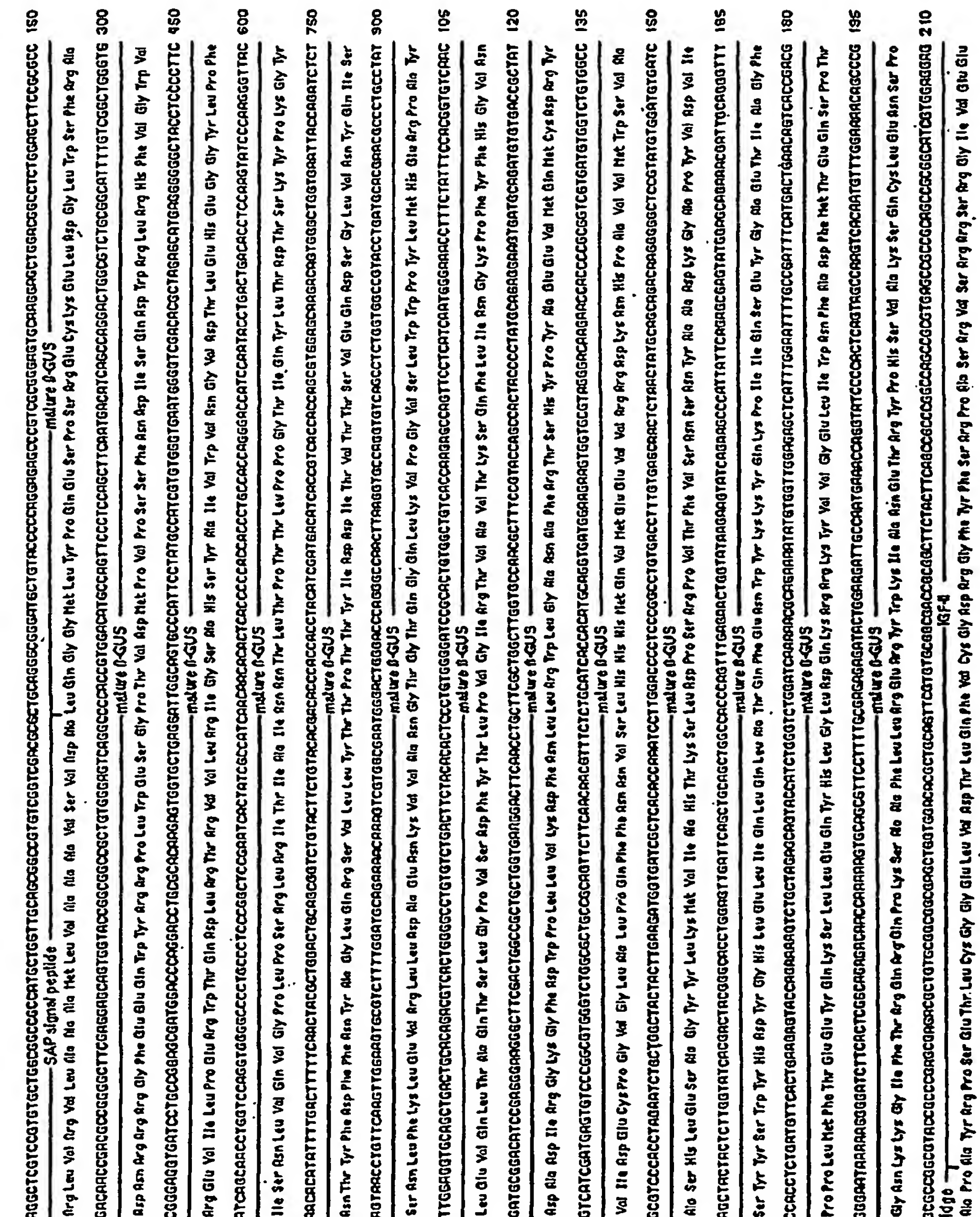


Figure 3.

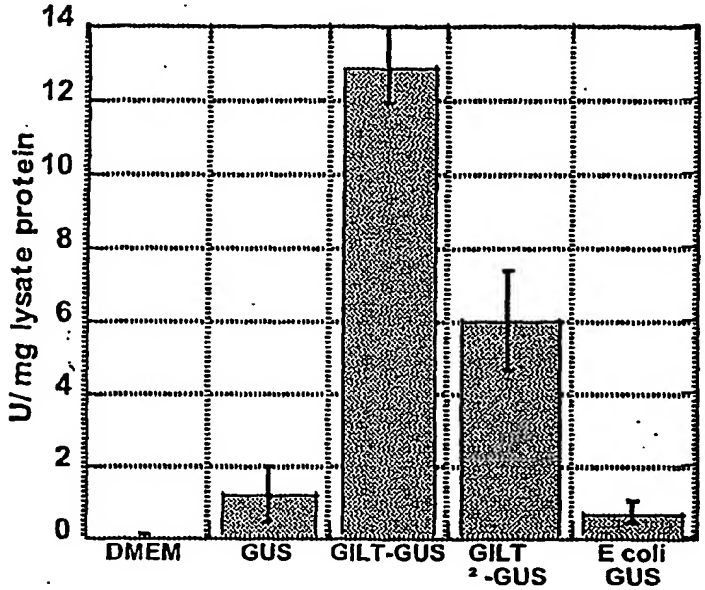

U/ mg lysate protein
14
12
10
8
6
4
2
0
DMEM
GUS
GILT-GUS
GILT ² -GUS
E coli GUS

Figure 4.

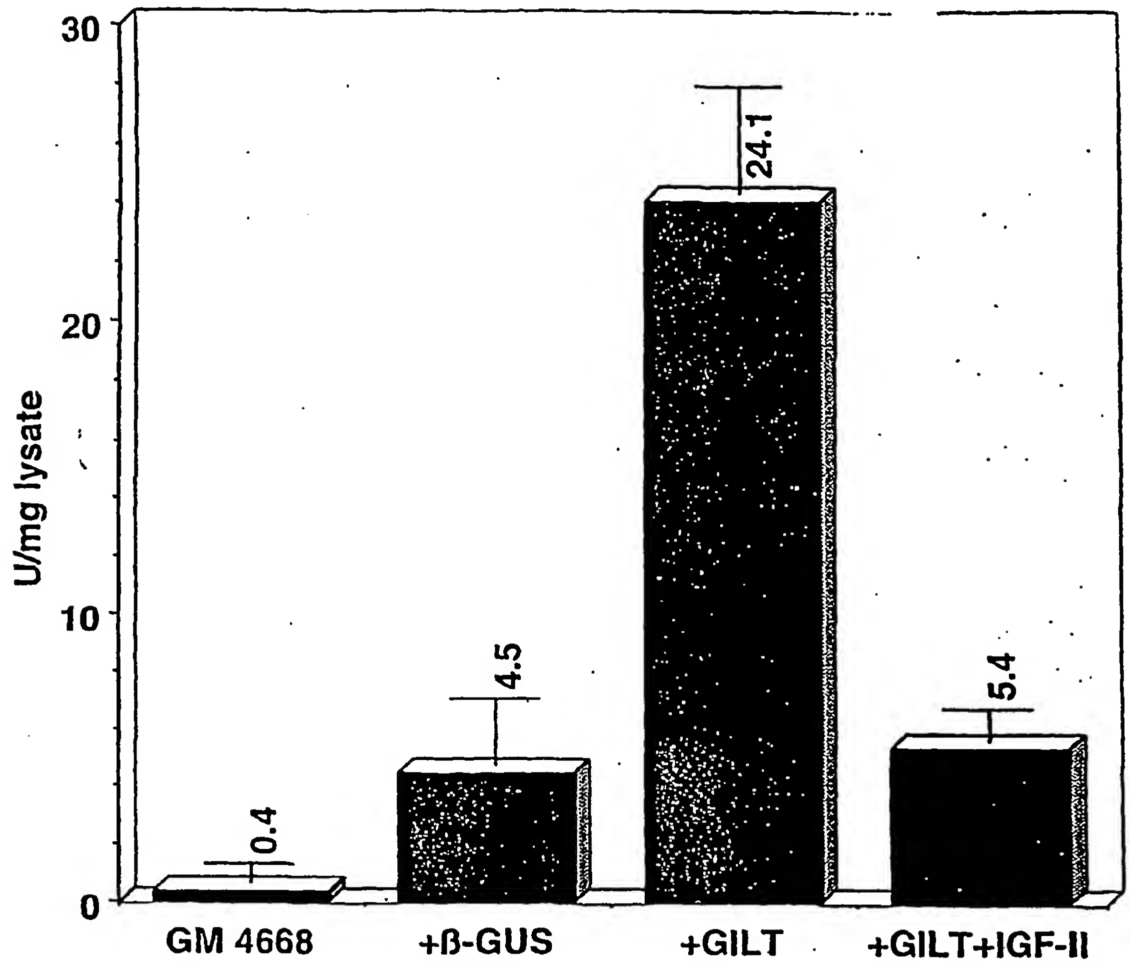


Figure 5.

Figure 6. Alignment of human IGF-I and IGF-II mature proteins showing location of domains.

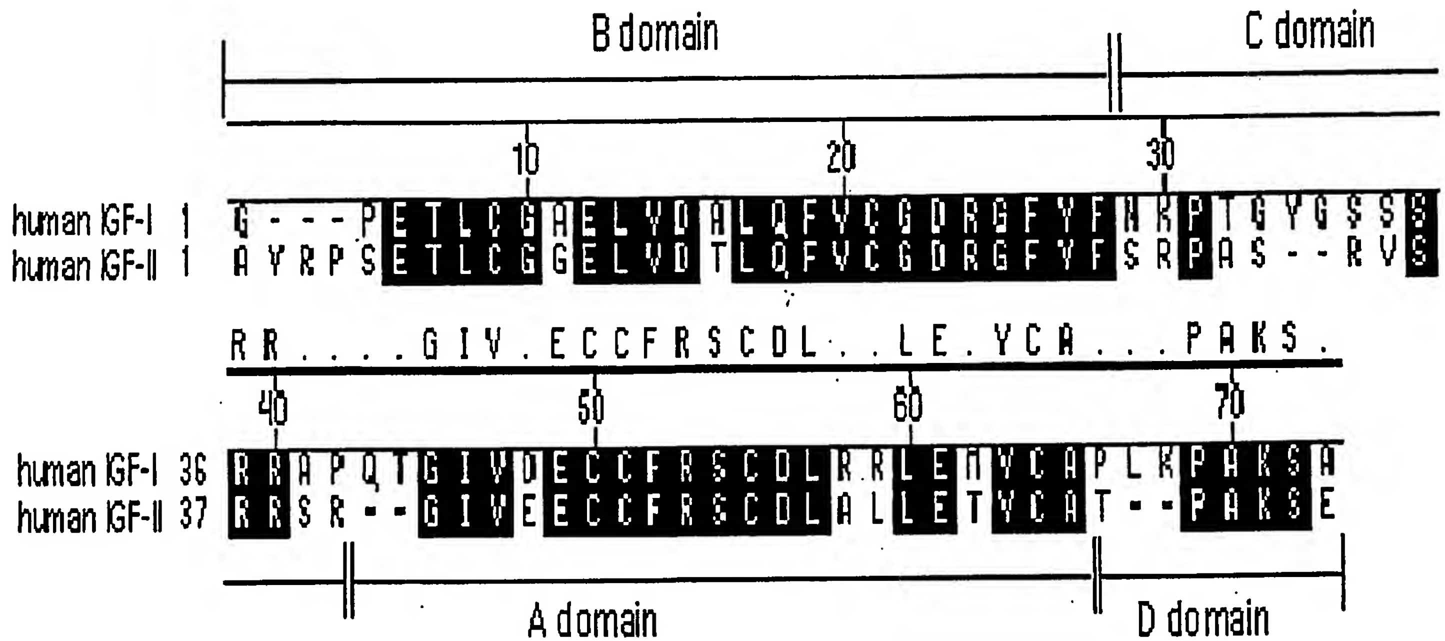
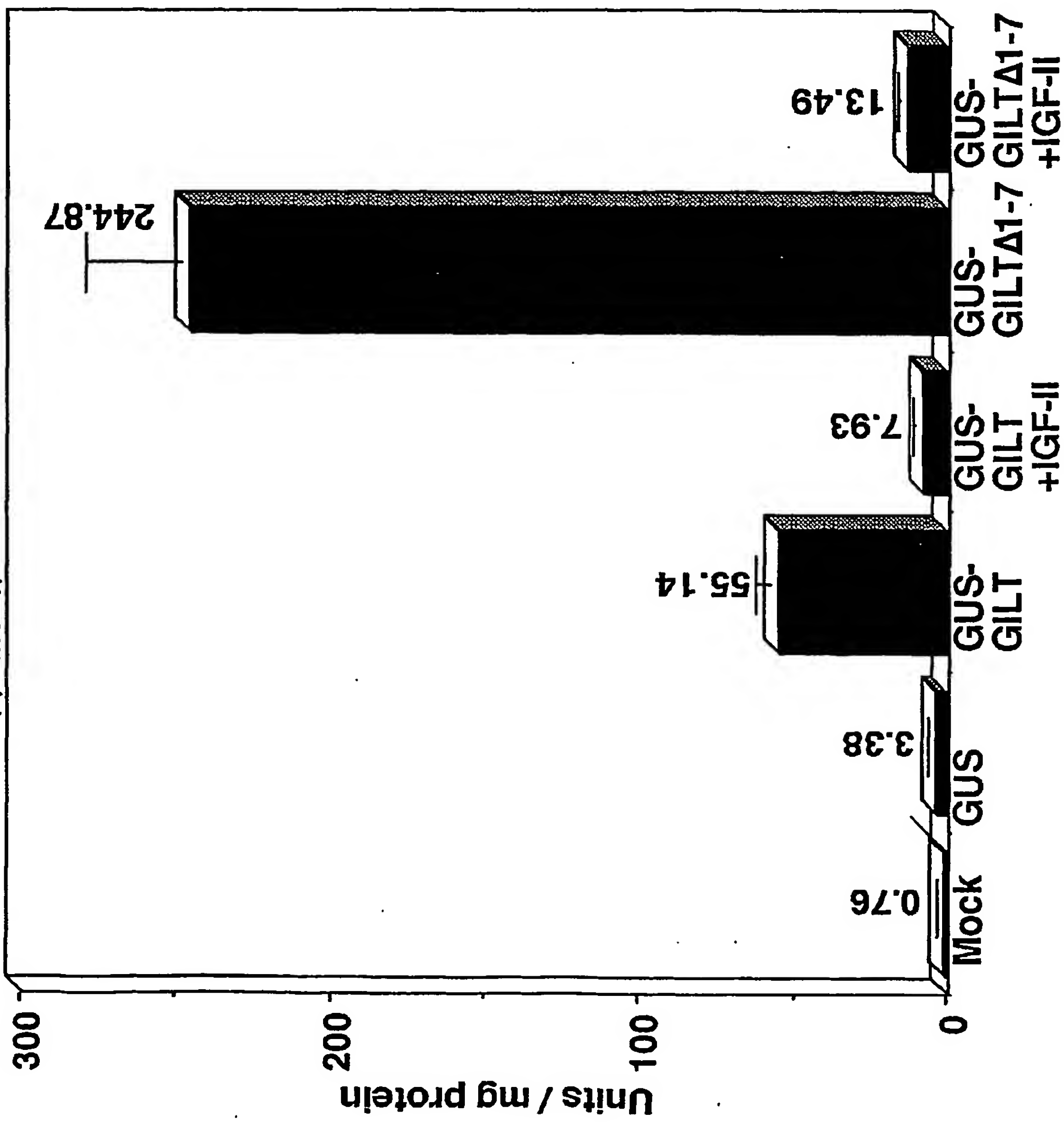


FIGURE 7


Units / mg protein
0
100
200
300
0.76
3.38
55.14
7.93
244.87
13.49
Mock
GUS
GUS-GILT
GUS-GILT +IGF-II
GUS-GILTΔ1-7
GUS-GILTΔ1-7 +IGF-II